

HANDBOOK OF FOOD BIOENGINEERING
VOLUME 6

GENETICALLY ENGINEERED FOODS



Edited by
Alina Maria Holban
Alexandru Mihai Grumezescu



Genetically Engineered Foods

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Handbook of Food Bioengineering,
Volume 6

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Alina Maria Holban
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Foreword

In the last 50 years an increasing number of modified and alternative foods have been developed using various tools of science, engineering, and biotechnology. The result is that today most of the available commercial food is somehow modified and improved, and made to look better, taste different, and be commercially attractive. These food products have entered in the domestic first and then the international markets, currently representing a great industry in most countries. Sometimes these products are considered as life-supporting alternatives, neither good nor bad, and sometimes they are just seen as luxury foods. In the context of a permanently growing population, changing climate, and strong anthropological influence, food resources became limited in large parts of the Earth. Obtaining a better and more resistant crop quickly and with improved nutritional value would represent the Holy Grail for the food industry. However, such a crop could pose negative effects on the environment and consumer health, as most of the current approaches involve the use of powerful and broad-spectrum of pesticides, genetic engineered plants and animals, or bioelements with unknown and difficult-to-predict effects. Numerous questions have emerged with the introduction of engineered foods, many of them pertaining to their safe use for human consumption and ecosystems, long-term expectations, benefits, challenges associated with their use, and most important, their economic impact.

The progress made in the food industry by the development of applicative engineering and biotechnologies is impressive and many of the advances are oriented to solve the world food crisis in a constantly increasing population: from genetic engineering to improved preservatives and advanced materials for innovative food quality control and packaging. In the present era, innovative technologies and state-of-the-art research progress has allowed the development of a new and rapidly changing food industry, able to bottom-up all known and accepted facts in the traditional food management. The huge amount of available information, many times is difficult to validate, and the variety of approaches, which could seem overwhelming and lead to misunderstandings, is yet a valuable resource of manipulation for the population as a whole.

The series entitled *Handbook of Food Bioengineering* brings together a comprehensive collection of volumes to reveal the most current progress and perspectives in the field of food engineering. The editors have selected the most interesting and intriguing topics, and have dissected them in 20 thematic volumes, allowing readers to find the description of

basic processes and also the up-to-date innovations in the field. Although the series is mainly dedicated to the engineering, research, and biotechnological sectors, a wide audience could benefit from this impressive and updated information on the food industry. This is because of the overall style of the book, outstanding authors of the chapters, numerous illustrations, images, and well-structured chapters, which are easy to understand. Nonetheless, the most novel approaches and technologies could be of a great relevance for researchers and engineers working in the field of bioengineering.

Current approaches, regulations, safety issues, and the perspective of innovative applications are highlighted and thoroughly dissected in this series. This work comes as a useful tool to understand where we are and where we are heading to in the food industry, while being amazed by the great variety of approaches and innovations, which constantly changes the idea of the “food of the future.”

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Series Preface

The food sector represents one of the most important industries in terms of extent, investment, and diversity. In a permanently changing society, dietary needs and preferences are widely variable. Along with offering a great technological support for innovative and appreciated products, the current food industry should also cover the basic needs of an ever-increasing population. In this context, engineering, research, and technology have been combined to offer sustainable solutions in the food industry for a healthy and satisfied population.

Massive progress is constantly being made in this dynamic field, but most of the recent information remains poorly revealed to the large population. This series emerged out of our need, and that of many others, to bring together the most relevant and innovative available approaches in the intriguing field of food bioengineering. In this work we present relevant aspects in a pertinent and easy-to-understand sequence, beginning with the basic aspects of food production and concluding with the most novel technologies and approaches for processing, preservation, and packaging. Hot topics, such as genetically modified foods, food additives, and foodborne diseases, are thoroughly dissected in dedicated volumes, which reveal the newest trends, current products, and applicable regulations.

While health and well-being are key drivers of the food industry, market forces strive for innovation throughout the complete food chain, including raw material/ingredient sourcing, food processing, quality control of finished products, and packaging. Scientists and industry stakeholders have already identified potential uses of new and highly investigated concepts, such as nanotechnology, in virtually every segment of the food industry, from agriculture (i.e., pesticide production and processing, fertilizer or vaccine delivery, animal and plant pathogen detection, and targeted genetic engineering) to food production and processing (i.e., encapsulation of flavor or odor enhancers, food textural or quality improvement, and new gelation- or viscosity-enhancing agents), food packaging (i.e., pathogen, physicochemical, and mechanical agents sensors; anticounterfeiting devices; UV protection; and the design of stronger, more impermeable polymer films), and nutrient supplements (i.e., nutraceuticals, higher stability and bioavailability of food bioactives, etc.).

The series entitled *Handbook of Food Bioengineering* comprises 20 thematic volumes; each volume presenting focused information on a particular topic discussed in 15 chapters each. The volumes and approached topics of this multivolume series are:

Volume 1: Food Biosynthesis

Volume 2: Food Bioconversion

Volume 3: Soft Chemistry and Food Fermentation

Volume 4: Ingredient Extraction by Physicochemical Methods in Food

Volume 5: Microbial Production of Food Ingredients and Additives

Volume 6: Genetically Engineered Foods

Volume 7: Natural and Artificial Flavoring Agents and Food Dyes

Volume 8: Therapeutic Foods

Volume 9: Food Packaging and Preservation

Volume 10: Microbial Contamination and Food Degradation

Volume 11: Diet, Microbiome, and Health

Volume 12: Impacts of Nanoscience on the Food Industry

Volume 13: Food Quality: Balancing Health and Disease

Volume 14: Advances in Biotechnology in the Food Industry

Volume 15: Foodborne Diseases

Volume 16: Food Control and Biosecurity

Volume 17: Alternative and Replacement Foods

Volume 18: Food Processing for Increased Quality and Consumption

Volume 19: Role of Material Science in Food Bioengineering

Volume 20: Biopolymers for Food Design

The series begins with a volume on *Food Biosynthesis*, which reveals the concept of food production through biological processes and also the main bioelements that could be involved in food production and processing. The second volume, *Food Bioconversion*, highlights aspects related to food modification in a biological manner. A key aspect of this volume is represented by waste bioconversion as a supportive approach in the current waste crisis and massive pollution of the planet Earth. In the third volume, *Soft Chemistry and Food Fermentation*, we

aim to discuss several aspects regarding not only to the varieties and impacts of fermentative processes, but also the range of chemical processes that mimic some biological processes in the context of the current and future biofood industry. Volume 4, *Ingredient Extraction by Physicochemical Methods in Food*, brings the readers into the world of ingredients and the methods that can be applied for their extraction and purification. Both traditional and most of the modern techniques can be found in dedicated chapters of this volume. On the other hand, in volume 5, *Microbial Production of Food Ingredients and Additives*, biological methods of ingredient production, emphasizing microbial processes, are revealed and discussed. In volume 6, *Genetically Engineered Foods*, the delicate subject of genetically engineered plants and animals to develop modified foods is thoroughly dissected. Further, in volume 7, *Natural and Artificial Flavoring Agents and Food Dyes*, another hot topic in food industry—flavoring and dyes—is scientifically commented and valuable examples of natural and artificial compounds are generously offered. Volume 8, *Therapeutic Foods*, reveals the most utilized and investigated foods with therapeutic values. Moreover, basic and future approaches for traditional and alternative medicine, utilizing medicinal foods, are presented here. In volume 9, *Food Packaging and Preservation*, the most recent, innovative, and interesting technologies and advances in food packaging, novel preservatives, and preservation methods are presented. On the other hand, important aspects in the field of *Microbial Contamination and Food Degradation* are shown in volume 10. Highly debated topics in modern society: *Diet, Microbiome, and Health* are significantly discussed in volume 11. Volume 12 highlights the *Impacts of Nanoscience on the Food Industry*, presenting the most recent advances in the field of applicative nanotechnology with great impacts on the food industry. Additionally, volume 13 entitled *Food Quality: Balancing Health and Disease* reveals the current knowledge and concerns regarding the influence of food quality on the overall health of population and potential food-related diseases. In volume 14, *Advances in Biotechnology in the Food Industry*, up-to-date information regarding the progress of biotechnology in the construction of the future food industry is revealed. Improved technologies, new concepts, and perspectives are highlighted in this work. The topic of *Foodborne Diseases* is also well documented within this series in volume 15. Moreover, *Food Control and Biosecurity* aspects, as well as current regulations and food safety concerns are discussed in the volume 16. In volume 17, *Alternative and Replacement Foods*, another broad-interest concept is reviewed. The use and research of traditional food alternatives currently gain increasing terrain and this quick emerging trend has a significant impact on the food industry. Another related hot topic, *Food Processing for Increased Quality and Consumption*, is considered in volume 18. The final two volumes rely on the massive progress made in material science and the great applicative impacts of this progress on the food industry. Volume 19, *Role of Material Science in Food Bioengineering*, offers a perspective and a scientific introduction in the science of engineered materials, with important applications in food research and technology. Finally, in volume 20, *Biopolymers for Food Design*, we discuss the advantages and challenges related to the development of improved and smart biopolymers for the food industry.

All 20 volumes of this comprehensive collection were carefully composed not only to offer basic knowledge for facilitating understanding of nonspecialist readers, but also to offer valuable information regarding the newest trends and advances in food engineering, which is useful for researchers and specialized readers. Each volume could be treated individually as a useful source of knowledge for a particular topic in the extensive field of food engineering or as a dedicated and explicit part of the whole series.

This series is primarily dedicated to scientists, academicians, engineers, industrial representatives, innovative technology representatives, medical doctors, and also to any nonspecialist reader willing to learn about the recent innovations and future perspectives in the dynamic field of food bioengineering.

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Preface for Volume 6: Genetically Engineered Foods

In recent years, the progress made in technology, industry, and bioengineering has led to the production of modified and completely new foods. Their properties, such as aspect and taste, but also availability, synthesis method, and economic impact have dictated the fate of a new food product. Moreover, nutritional properties and impact on consumers' health are the key parameters to be considered in their design. Genetically engineered foods have been considered controversial products since many years. Recent scientific evidence in genetic engineering have allowed the production of better crops, more resistant animals, and overall improved raw materials for an advanced food industry. The concerns related to the impact of genetically engineered foods on human health, biodiversity, and the future of life, as we know it today still remain, and numerous unknown and unpredicted aspects, which are constantly emerging, promote this field with precaution.

In context to the food crisis and increasing globe population, methods able to obtain better nutrition and basic food products faster, with increased performance and wider availability, such genetic engineering, are intensively investigated nowadays. The potential of genetic engineering in the future food industry has multiple uses: to ensure a basic life support, to offer an alternative for individuals with very specific food requirements, and to simply differentiate the classical food concept.

The aim of this book was to bring together the most recent progress achieved in the field of genetically engineered foods, empathizing the current approaches, actual concerns in the biotechnological and food industry fields, and success of very recent technologies. An updated overview regarding where we are situated in terms of genetically engineered foods and where we are heading to, can be also exploited within this book.

The volume contains 15 chapters prepared by outstanding authors from Romania, Denmark, United Kingdom, Iran, Pakistan, Germany, India, Nigeria, Brazil, and Turkey.

The selected manuscripts are clearly illustrated and contain accessible information for a wide audience, especially food scientists, engineers, biotechnologists, biochemists, molecular biologists, geneticists, industrial companies and also for any reader interested in learning about the most interesting and recent advances in the field of genetically engineered foods.

Chapter 1, *Genetic Engineered Organisms (Plants and Animals)*, prepared by Khalid et al., introduces readers with the basic concepts of genetically engineered plants and animals for a sustainable food industry. Their study highlights the endeavors and advances in plants and animals genetic engineering achieved by scientists globally by utilizing modern biotechnological approaches. Future prospects and risk assessment of the genetically engineered organisms (plants and animals) have also been overviewed in this chapter.

Chapter 2, *Sugar and Sugar Alcohol Production in Genetically Modified Cyanobacteria*, prepared by Frigaard, offers an updated overview regarding genetically engineered cyanobacteria to increase the diversity and yield of the produced sugars. Sucrose, fructose, glucose, glycerol, erythritol, and mannitol have been produced in genetically engineered cyanobacteria, although the yields need to be improved to have practical significance.

In **Chapter 3**, *Genetically Modified Plants as Sustainable and Economic Sources for RUTFs*, Dayani and Sabzalian are trying to elucidate the capacity of genetically engineered plants as sustainable and low-cost sources for supplying ready-to-use therapeutic foods (RUTFs) ingredients. The malnutrition concept, the current trends and standards in formulating the RUTFs, and the contribution of genetic engineering for achieving nutritional self-sustainability among hunger- and malnutrition-stricken populations by advancing nutritional-rich plant foods are discussed within this work.

Chapter 4, *Approved Genetically Engineered Foods: Types, Properties, and Economic Concerns*, written by Elena et al., reviews the advantages and disadvantages of genetically modified foods designated for human consumption and dissect economic concerns associated with their diversity and use.

In **Chapter 5**, *Influence of Feed From Genetically Modified Plants on the Composition and Quality of Foods of Animal Origin*, Flachowsky analyzed animal health and welfare, to follow the ways of transgenic DNA and the newly expressed proteins, and assessed the influence of genetically modified-plants (GMP) on the composition and quality of food of animal origin, such as milk, eggs, meat, and fish. It seems that feeds from GMP of the 1st generation does not significantly influence the composition and quality of food of animal origin and can be considered as substantial equivalent to their isogenic counterparts.

Chapter 6, *Genetically Modified Microorganisms: Harmful or Helpful?*, prepared by Csutak and Sarbu, deals with some of the most relevant aspects regarding the main results obtained in the food industry by using genetically modified lactic acid bacteria and yeasts. Authors are analyzing the benefits and the harmful issues, offering a glimpse of the current legislation and public concerns related to this subject.

In **Chapter 7**, *Techniques for Production and Quality Assessment of Genetically Modified Foods*, Jain et al. provide a detailed account on the types of genetically modified foods, various methods of their production, and methods for assessing the safety profile of

genetically modified foods. The major challenges witnessed in predicting and assessing unintended adverse effects of genetically modified foods include intense imbalances between highly advanced analytical technologies and limited abilities to interpret the results and their correlation with the health effects that occur due to consumption of genetically modified foods. Therefore, a number of approaches have been developed for assessing the occurrence and significance of unintended health effects based on both targeted and profiled information using a range of toxicological, metabolic, and epidemiological sciences.

Chapter 8, *Awareness and Utilization of Genetically Modified Foods in Nigeria*, written by Agaviezor, aims to increase the awareness regarding the utilization of genetically modified foods in Nigeria and to discuss whether genetically modified foods could be used to solve the problem of food insecurity in this highly food-deprived country.

Chapter 9, *Biosensors as Advanced Device for the Transgenic Plants and Food and Detection*, Sousa et al. offer an updated overview of biosensors, namely immunosensors and genosensors used as analytical techniques for the assessment of transgenic in plants and foodstuffs. Several critical aspects will be discussed here, namely the biosensor design and its construction, the transducer platform selection, biosensor nanostructuration (with nanomaterials and nanoparticles) to increase the sensitivity, and the reasonable manner of choice detection methods.

Chapter 10, *Genetically Engineered Food Crops to Abiotic Stress Tolerance*, written by dos Reis et al., aims to present the recent studies about the genetically engineered crops to abiotic stress resistance, highlighting the main food sources. The growth and yield of crops are greatly influenced by environmental stresses once plants are sessile. Furthermore, an aggravating factor is the global climate changes that can compromise the food security in the world. In the last 30 years, new methodologies were developed to allow the production of genetically modified crops with an improvement of tolerance to abiotic stresses. These modified crops not just survive stress, but also show good yields under stress conditions, and these aspects are discussed within this work.

In **Chapter 11**, *Detection of Prevented DNA Damage by Therapeutic Foods*, Dogan-Topal et al. discuss about the modified and therapeutic foods revealing the assay strategies employed for the detection of DNA damage. Mutations play a significant role both in cancer initiation and in other stages of the carcinogenic process. Oxidative stress, involves oxidation of biomolecules by reactive oxygen species, has been strongly correlated to the degenerative diseases, especially cancer. The therapeutic foods, such as those rich in vitamins, carotenoids, flavonoids, phenolic acids are gaining attention as potential protectors against the degenerative disorder especially cardiovascular disease and cancer.

Chapter 12, *Genetically Engineered Crops: Opportunities, Constraints, and Food Security at a Glance of Human Health, Environmental Impact, and Food Quality*, prepared by Dawkar

et al., describes genetic modification (GM) as an incredible technology among the top ten technologies developed in the 20th century. People believe that GM technology will answer the serious concern of future demand for food to feed millions of people. Overall, it was demonstrated that adoption of GM crops increases farmers' income and proved to be vital in drug production. But we have to be very careful before releasing GM crops in the environment because once planted the GM plant in the environment, there won't be a turn back.

In **Chapter 13**, *Safety and Risk Assessment of Food From Genetically Engineered Crops and Animals: The Challenges*, van Rijssen and Morris highlighted the safety and risk from the genetically engineered (GE) food and the genetically modified organisms. The current focus is on determining whether intended and/or unintended effects have arisen with genetic modification (engineering). This chapter aims to make a distinction between the concept of safety and risk. Zero risk is not possible. International bodies have played significant roles in guiding the interpretation of these concepts. Analysis and interpretation of the composition of GE-food is key to the targeted approach of assessment. The understanding of environmental effects on component concentration are becoming clear through the metaanalysis of crop data. The current focus is globally on the value of toxicology studies with rodents, nontarget approach of 'omics', stacking of traits and the need for extensive safety studies for food produced using gene editing techniques.

Chapter 14, *Identification of Genetically Modified Foods*, prepared by Iordache et al., presents and discusses the advantages and disadvantages of molecular detection methods [based on indirect (immunologic) and direct (genetic) approaches] and the new technologies that offer high-throughput information regarding the characteristics of genetically modified foods. Furthermore, the sample collection and validation criteria are presented highlight the crucial role in obtaining the reliable results.

In **Chapter 15**, *Methods for Plant Genetic Modification*, Bleotu et al. discuss the plant genetic manipulation methods, which can be divided into those requiring in planta transformation and tissue culture. Both nongenetic engineering and genetic engineering approaches currently developed for plant genetic modifications are presented here. Advantages and disadvantages both in terms of getting the modified plants and in the effects they may have on human health are also discussed and a perspective for future genetically modified crops is drafted.

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Genetic Engineered Organisms (Plants and Animals)

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1 Genetic Engineering

Genetic engineering is the process of introducing foreign DNA into an organism's genome. A chimeric DNA with genomic pieces from two different sources is called recombinant DNA and the process is referred to as recombinant DNA technology. "Genetically engineered" (GE), "genetically modified" (GM), or "transgenic" are all terms that can be used in this context. A GM organism will carry one or more traits that were not already present in the original organism (Rabbani, 2003). It can be performed through gene transfer of soil bacterium, *Agrobacterium tumefaciens* using a plasmid vector (Smith and Townsend, 1907), having additional genes, or the bombardment of gold particles (Foster et al., 2006). A variety of transgenic plants modified for traits, such as insect resistance, herbicide tolerance, drought resistance, salinity resistance, and temperature resistance have been developed. Foods where traits have been improved to make them more desirable in society are referred to as GM foods. GM foods are becoming increasingly popular throughout the world; however, they are a topic of debate among many people.

2 Purpose of Genetically Engineered Organisms

The development, production, and marketing of GM foods and animals were initially performed due to concerns over crop protection. Both producers and consumers believed that GM foods could be beneficial, nutritious, and helpful for overcoming the future food shortage. In addition to increased plant protection from diseases, insects, and herbicides, other reasons for the development of GM animals and plants included the need for abiotic stress tolerance, enhanced crop productivity, enhanced meat production (poultry and fish), pharmacogenomics, and edible vaccines (Lemaux, 2008).

Malnutrition is predominant in third world countries where the nutritional requirements of the people tend to be met by only one crop product. Approximately 800 million adults and 250 million children are seriously malnourished in underdeveloped countries (McMichael et al., 2005). GM crops were commercialized to reduce poverty in underdeveloped countries (i.e., provide financial benefits to rural incomes) and provide crops with high nutritional values to overcome nutrient deficiencies in the poor (Farre et al., 2011). For example, blindness due to vitamin A deficiency is very frequent in third world countries, therefore the Swiss Federal Institute of Technology developed a GM rice variety (Golden Rice) with enhanced levels of vitamin A (Potrykus, 2001).

Similarly, the purpose of producing GM animals can be divided into two groups: food and nonfood products. Animals included in the first group have been modified for economic benefits (e.g., enhanced meat and milk production, increased rates of growth, increased efficiency of feeding, sustained survival, and immunity against environmentally acquired infections). The second class of GM animals includes those that are able to produce specific substances in their milk, meat, blood, and eggs (Gruere, 2006). Coping with food scarcity has become an enormous challenge due to the rapidly increasing global population, which may reach ~9.7 billion in 2050. Therefore how can we feed tomorrow's population with yesterday's limited resources? Transgenic technology is therefore essential to boost the food supply for the growing population.

3 *Genetic Engineering Versus Hybridization Techniques*

Plant and animal breeding is a method of producing offspring with desired traits using conventional techniques, such as hybridization/sexual mating between two different species (Hayward et al., 2012). Conversely, genetic engineering is a fast and targeted method, which primarily adds or modifies the expression of genes to achieve desired traits (Snow et al., 2005).

Selective breeding has played a vital role in crop and animal improvement since Antediluvian times. Agriculture started with the breeding, selection of wild grasses, and precursor advancement of current staples, such as rice, maize, and wheat (Baloch et al., 2010; Comertpay et al., 2012). Conventional breeding, the slow rate of development of new crop varieties, and the curb of fertility barriers that prevented the hybridization of the same or closely associated species, have all significantly contributed to the development of high-yielding crop cultivars. However, improvements in crop cultivars due to genetic engineering technologies have gained momentum in first and second world countries over time (Bakhsh et al., 2009; Hussain, 2002).

Plant breeding is traditionally performed by the direct transfer of pollen from male organs to female organs. Similarly, animal breeding is commonly achieved through sexual mating

of two different animals (Brown and Caligari, 2011). However, these crossbreeding methods have limitations in that they can only occur between sexually compatible or closely related species, and they require long periods of time (i.e., from fertilization to adulthood). Moreover, the sustainability of hybrid offspring is a controversial issue (Murphy, 2007).

Genetic engineering is more specific than conventional breeding and it bypasses the crossbreeding generation time. One potential advantage of GE over classical breeding is knowledge of the gene being inserted, whereas a few studies have reported the introduction of unwanted genes using classical breeding. Another major difference between these two approaches is the role of GM organisms in pharmacology and edible vaccine development. Such products cannot be achieved through conventional breeding. Although genetic engineering is not a substitute for conventional breeding, it can expressively contribute to plant breeding by producing further genetic diversity (Xu, 2010). However, there are uncertainties about where the transgenes are inserted in the genome and transgenic expression makes GE less precise than originally thought. It is now time to evenly assess the studies related to the benefits and possible dangers of genetic engineering in comparison with other crop development techniques (Gepts, 2002).

4 Genetically Modified Plants

At the beginning of the 19th century, Smith and Townsend (1907) found that a bacteria (now called *A. tumefaciens*) was responsible for the formation of crown gall disease in plants. Many researchers have since concentrated their efforts on this bacterium to thoroughly investigate the molecular basis of the induction, development, and proliferation of crown gall disease and understand oncogenesis in more detail. Discovery of the “oncogenic gene transfer mechanism from *Agrobacterium* into plants” refreshed research interest in this field and it was perceived that this transfer system might be a tool for the genetic engineering of plants (Hooykaas and Schilperoort, 1992). The genetic engineering of plants was considered to be an advanced research area in the 1970s and 1980s and it was constantly used for crop improvement. Therefore various new plant varieties were developed in the last decades of the 19th century (Duvick, 2003).

China introduced the first GM tobacco plant (*Nicotiana tabacum* L.) in 1983, which was modified with genes for antibiotic resistance (Ganapathi et al., 2004). However, Flavr Savr tomatoes are considered as the first commercial GM plant despite their limited commercial success in the United States; although many modifications of tomatoes and eggplants have since been investigated (Hautea and Escaler, 2004). A GM variety of soybean named “Monsanto’s Roundup Ready soybean” was permitted for commercial utilization in 1996 (Samardzic and Timotijevic, 2010), and the first transgenic wheat and rye hybrids were developed by scientific breeders in 1876 (Finkel, 2008). Wheat was also the first transgenic cereal to be developed from three parental species (Bates et al., 2005).

In 1998 researchers from Aberdeen University (Scotland) injected potatoes with an insect resistant gene isolated from the snowdrop plant and found that these potatoes were resistant to field rats and insects (Ewen and Pusztai, 1999). Similarly, herbicide tolerant GM crops were developed in 1999; however, opponents criticized these GM foods stating that they were potentially harmful to nearby crops and natural pollinators (Uzogara, 2000). Regardless of the debate surrounding GM plants, genetic engineering advanced to a level where about 10 million farmers in 22 countries planted 252 million acres of transgenic crops (2006). The United States, Brazil, Canada, Argentina, India, South Africa, Paraguay, and China cultivated 97% of these crops (Lemaux, 2008). The need to improve crop yields and eliminate usage of pesticides has seen the food crop industry take a vested interest in the production of GM crops over the last 10 years. Successful examples where transgenes have been used for insect resistance and herbicide tolerance are seen in soybeans, corn, cotton, canola, and alfalfa, while other crops, such as sweet potatoes have been modified against environmental stresses (i.e., salinity and drought) (Ahmad et al., 2012).

Farmers in trading and developing countries rapidly adopted transgenic crops between 1996 and 2015 because of their potential benefits (Table 1.1). The global area under GM crops has increased spectacularly over the last 17 years, thanks to this environmentally friendly technology (Qaim, 2009).

Current studies show that GM agriculture has received considerable consideration as the area under cultivation increased from 1.7 million hectares in 1996 to 160.4 million hectares in 2012, predominantly via the introduction GM maize, corn, canola, and soybeans. Successful field trials of GM crops has led to 14 million farmers in 25 different countries intending to grow GM crops. In India, 75% of the total cultivated area is specified for transgenic cotton cultivation. Some GM crops (i.e., insecticide and herbicide resistant) have been introduced commercially and crops with additional traits are expected to be released in the near future (Shahid et al., 2016; Yaqoob et al., 2016).

Modern biotechnology and GM crops can be beneficial to combat food scarcity in developing countries as farmers can produce higher crop yields within a shorter time. Increased drought resistance, prolonged shelf life, valuable food quality, and depleted pesticide use are also advantages of GM crops that are beneficial across the world. Supporters of GM plants recognize the beneficial impacts of biotechnology in improving conventional plant breeding techniques (Azadi and Ho, 2010). Furthermore, GM crops can be helpful in planning new foods with particular micronutrients or relevant properties.

In the future it will be necessary to enhance agricultural productivity to meet the demands of an increasing population and cope with limited natural resources and changing climatic conditions.

Table 1.1: Common examples of transgenic crops, their target traits, involved methodologies, and transgenes (Ricroch and Henard-Damave, 2015).

Species	Target Traits	Transgenic Genes	Methodologies	References
Apple	Scab resistance	<i>HcrVf2</i>	Cisgenesis	Vanblaerea et al. (2011)
Potato	Late blight resistance	Potato <i>RB</i>	Transgenesis	Bradeen et al. (2009)
Banana	Parasitic nematode and weevil resistance	Cystatin (<i>OcdeltaD86</i>)	Transgenesis	http://www.banana.go.ug/
Chestnut	Fungal blight resistance	<i>NpII</i> gene and chitinase	Transgenesis and embryonic suspension-based transformation	Maynard et al. (2008); Andrade et al. (2009)
Eggplant	Brinjal fruit and shoot borer resistance	<i>cry1AC</i>	Agrobacterium-mediated transformation	Pal et al. (2009)
Rice	Lepidopteran pests tolerance	<i>cry1Ab</i>	Agrobacterium-mediated transformation	Shu et al. (2000)
Sugar cane	Stem borer resistance	BTK HD-1 <i>cryIAb</i>	Transgenesis	Arencibia et al. (1997)
Sweet potato	Weevil (beetle) resistance	Delta-endotoxin gene	Transgenesis	Moran et al. (1998)
Tomato	Against bacterial spot disease	<i>Bs2</i>	Transgenesis	Horvath et al. (2012)
Walnut	Against bacterial, viral, and fungal attacks	NA	Grafting on GM root-stock on wild type Scion	Haroldsen et al. (2012)
Tobacco	Fungal attack (<i>Verticillium dahliae</i>) resistance	Thaumatococin-like protein gene	Transgenesis	Munis et al. (2010)
Wheat	Fungal attack (<i>Fusarium graminearum</i>) resistance	Class II chitinase gene	Transgenesis	Shin et al. (2008)
Corn	Drought resistance	Gene-silencing approach to alter ethylene biosynthesis	RNAi	Habben et al. (2014)
Soybean	Drought resistance	Overexpression of <i>Hahb-4</i> gene to inhibit ethylene induced senescence	Transgenesis	Manavella et al. (2006)

4.1 *Bacillus thuringiensis* (Bt) Berliner Crops: A Blueprint to the GM Plantation

The common soil bacterium, *B. thuringiensis* (Bt), possess a gene encoding a Cry protein that is toxic to lepidopteran larvae (caterpillars) but harmless to humans, therefore this gene has been inserted into the cotton plant genome. A cotton plant with the Bt gene is referred to as Bt cotton and it is resistant to attack from insects, such as the pink bollworm (Kumar et al., 2008). The Cry protein binds to specific molecular receptors on the caterpillar's gut lining after ingestion and creates a puncture in the insect's gut, resulting in the quick death of the larvae. The specific mechanism of the Cry protein means that it specifically target pests and cannot harm beneficial pollinator or other phytophilous insects (Yaqoob et al., 2016). Hence bioengineered crops are specific for their natural enemies when compared with conventional insecticides (Gould, 1998).

Now the time has come to celebrate 30th anniversary of transgenic technology in order to develop the genetically modified plants. Insects relentlessly affect the production of agricultural crops worldwide. The enhanced crop production when using insect-resistance genes (Bt) and the economic benefits to farmers has seen an increase in the commercial release of almost all transgenic crops expressing Bt genes. However, insect-resistant non-Bt crops have not been reported in either large-scale field trials or commercial release. RNAi technology and integration of the genes belonging to proteinase inhibitors and lectin are presumed to be alternative options for the development of pest-resistant crops; however, these technologies are still in their formative years. Regardless of the economic benefits of Bt crops, nonominous reports of vertical and horizontal gene flow, antibiotic resistance, and the fact that Bt proteins have the ability to degrade in the soil (degradation kinetics) means that Bt crops still receive criticism from consumers, research institutions, and nongovernmental organizations who perceive that these crops have negative impacts on nontarget organisms (NTOs) (Bakhsh et al., 2015).

5 Techniques to Develop Genetically Modified Plants

The progress in tissue culture and plant transformation technologies resulted in the production of transgenic crops, which opened up a new field for plant biotechnologists. The manufacture of new chimeric genes extended plant transgenic technologies (Fraleigh et al., 1983; Herrera-Estrella et al., 1983), plant expression vectors (Bevan, 1984; Hoekema et al., 1983), DNA transfer (Draper et al., 1982; Fromm et al., 1985; Stanford et al., 1987), and plant regeneration and transformation methods (Gordon-Kamm et al., 1990; Shimamoto et al., 1989; Zambryski et al., 1983).

Transformation technologies can be divided into direct or indirect methods. Direct methods do not utilize bacterial cells as mediators and the most commonly used methods are

microprojectile bombardment or protoplast transformation. Indirect methods introduce a binary vector (carrying the gene of interest) into a plant cell using *A. tumefaciens* or *Agrobacterium rhizogenes*. *Agrobacterium* and microprojectile bombardments are the most widely used methods for transformation (Dai et al., 2001), although other methods, such as infiltration, silicon carbide fiber-mediated transformation, microinjection, liposome-mediated transformation, and transformation by the pollen-tube pathway (PTP) are also used (Newell, 2000; Patnaik and Khurana, 2001; Rao et al., 2009; Songstad et al., 1995; Vasil, 1994; Zhu et al., 1993). Techniques employed for the construction of GM plants are described in the following sections.

5.1 Recombinant DNA Technology

Recombinant DNA technology is essentially used to improve plants and it has been shown to produce the most effective results. An extraordinary advancement was made in 1990 by researchers at Monsanto (United States) who modified *Bt* genes (*cryIAb* and *cryIAc*) to improve plant yields (Perlak et al., 1991). Expression of *cryIAc* in cotton and *cry3Aa* in potato enhanced the crops defenses against lepidopterans and coleopteran pests, respectively. Immunological analysis and insect feeding bioassays showed that the *Bt* proteins were the most important proteins in the transgenic plants. The resistance genes were transmitted to the progeny in a Mendelian fashion and the offspring were apparently normal. In field experiment, these modified plants showed enhanced protection against cotton bollworm (*Helicoverpa zea*) (*Pectinophora zea*) and pink bollworm (*Pectinophora gossypiella*) (Perlak et al., 1991). *Bt* cotton was created through the addition of genes encoding toxin-producing Cry proteins (endotoxin). In this process Cry toxins were generated due to the high pH level within the insect's stomach when they ingested the plant. Cry molecules adhere to cadherin-like proteins on brush-border cells (Caroll et al., 1997). The inner sheet of the brush-border membrane separates the body from the gut permitting the availability of nutrients. Cry toxin molecules adhere to exact positions on the cadherin-like proteins, which in turn create ion channels that permit the movement of K ions. K ions are necessary for survival but when the regulation is lost and levels elevate it leads to the demise of the cells. Loss of these cells creates gaps in the brush border membrane (Schnepf, 1998).

5.2 RNAi (Silencing)

RNA interference (RNAi), which encompasses microRNA and exogenously derived small interfering RNA, induces the degradation of complementary mRNA (Gunawardane et al., 2007). There are three possible pathways for silencing specific genes in plants using RNAi. Signals can be enlarged and transmitted between cells during silencing, and may even be self-regulated by feedback mechanisms. These mechanisms have vast biological functions, such as protection against viruses, deactivation of chromatin into heterochromatin,

and regulation of gene expression. These mechanisms allow us to examine the functional diversity in genetic and epigenetic mechanisms of genome control.

Infiltration, silicon carbide fiber-mediated transformation, electroporation of cells and tissues, microinjection, liposome-mediated transformation, and transformation via the PTP can also be used for silencing (Newell, 2000; Patnaik and Khurana, 2001; Rao et al., 2009; Songstad et al., 1995; Vasil, 1994; Zhu et al., 1993).

5.3 Tissue Culture and Transformation

5.3.1 Direct micro projection

In the direct method bacterial cells are not used as intermediaries, and the most practical methods are microprojectile bombardment or protoplast transformation (Dai et al., 2001). Microprojectile bombardment (i.e., particle bombardment, gene guns, or biolistics) is the most commonly used method for DNA delivery to the plant genome. These methods were used in the early 1980s (Paszkowski et al., 1984; Shillito, 1999), particularly for cash crops as they were exterior to the hosts range of *Agrobacterium* and therefore insensitive to *Agrobacterium*-mediated transformation (Vasil, 2005). Cereal crops, such as corn, wheat, and rice have been transformed using these methods. There are some restrictions to particle bombardment when compared with *Agrobacterium*-mediated transformation. These restrictions include incorporation of multiple copies of the transgene at a single chromosomal position, gene rearrangement, and integration of the transgene at multiple insertion sites. These numerous replicates have sometimes been associated with transgene silencing in descendants (Yao et al., 2006).

5.3.2 Indirect micro projection

In indirect microprojection binary vectors carrying the desired gene are introduced into the plant cell by *A. tumefaciens* or *A. rhizogenes*. (Dai et al., 2001).

5.4 Infiltration

The infiltration method of transformation adds bacterial strains into plant parts (e.g., root or shoot tips) with high metabolic activities (i.e., meiosis and mitosis). This method does not require in vitro culture and it has primarily been used for the transformation of *Arabidopsis thaliana* and is considered as a key gene delivery method in this species. However, infiltration has not been successful for the transformation of species, such as Chinese cabbage (Liu et al., 1998). Bechtold et al. (1993) introduced vacuum infiltration for the transformation of *Arabidopsis*. In this method, juvenile plants were placed upside down in a beaker filled with sucrose solution (5%) and a bacterial strain of *A. tumefaciens* in a vacuum chamber (0.05 bar for a few minutes). The younger plants were submerged in such a way that their inflorescence was completely submerged in the

solution. Seeds were later collected from these plants and sown in selective media under aseptic conditions. Under ideal conditions, 0.5%–4% of transgenic seeds were harvested and this percentage increased over the next 5 years. Clough and Bent (1998) improved this protocol by optimizing the concentration of sucrose and enhancing the bacteria using Silwet L-19 surfactant. This classical protocol has now become the most widely used method for the transformation of *Arabidopsis* (Chung et al., 2000; Ye et al., 1999). Chung et al. (2000) introduced a modified version of this protocol, in which the vacuum was not required for infiltration and this method is useful for the transformation of plants that are larger than *Arabidopsis*. In this modified method plants are immersed or sprayed with a bacterial suspension after the first leaf is removed. Transformation by omitting the vacuum gave the best result when spraying plants with bacterial suspension compared with the classical method with a vacuum chamber.

5.5 Silicon Carbide-Mediated Transformation

Silicon carbide-mediated transformation (SCMT) is considered as the main transformation method for maize. This is a simple, quick, and inexpensive plant transformation method that is useful for many plant materials. It is a good alternative method as it does not cause the difficulties seen in *Agrobacterium*-mediated plant transformation and is especially useful in the case of monocots or when a gene gun is unavailable. SCMT also has the potential to be used for the large-scale commercial transformation of maize (Bullock et al., 2001). Silicon carbide is a single crystal of organic silica minerals with a length of 10–80 mm and a diameter of 0.6 mm, thus having an elongated shape with a high expandability value. However, the vessel shape, vortexing parameters, fiber size, cell wall thickness, and nature of the plant material can all affect the efficiency of SCMT. In this method, silicon carbide fibers are vigorously mixed with plasmid DNA and other plant tissues (e.g., callus, cell clumps, or immature embryos) using a blender, a shaker, or by vortexing. DNA-coated fibers then enter the plant cells through holes created during the collision of fibers and plant cells (Kaeppler et al., 1990, 1992; Wang et al., 1995).

SCMT has been used to derive transgenic forms, cell colonies, or plants in maize (Bullock et al., 2001; Frame et al., 1994; Kaeppler et al., 1992; Petolino et al., 2000; Wang et al., 1995), rice (Nagatani et al., 1997), wheat (Brisibe et al., 2000; Serik et al., 1996), tobacco (Kaeppler et al., 1990), *Lolium perenne*, *Festuca arundinacea*, *Lolium multiflorum*, and *Agrostis stolonifera* (Dalton et al., 1997).

The main disadvantages and limitations of SCMT are (Songstad et al., 1995):

1. The transformation efficiency is lower than other methods of transformation.
2. The method can be hazardous to health in the lab as breathing in the fibers causes serious sickness (especially if asbestos is inhaled).

3. This technique damages cells, which negatively affects their capacity to regenerate.

5.6 The Pollen-Tube Pathway Method

The unknown DNA can be used to cut styles into small sizes after pollination. DNA penetrates into the female reproductive part by curving down the pollen tube (penetration into the pollen tube is why this technique is called the PTP method) and it was first reported for the transformation of rice (Luo and Wa, 1988). The PTP method has also been used in crops, such as wheat (Mu et al., 1999), soybean (Hu and Wang, 1999), and watermelon (Chen et al., 1998). The active material (bacteria) or circular DNA (plasmid) can also be injected into mother pollen cells before meiosis and the foreign or invader DNA will then be incorporated with the genome of the haploid cells. This type of integration occurs in rye (De la Pena et al., 1987).

5.7 Liposomes

Liposomes are minute, sphere-shaped vesicles that are able to carry a variety of molecules, including DNA. In protoplasts the transfection (lipofection) occurs by membrane fusion and endocytosis. The liposomes are transferred through pores when pollen grains are transformed. This technique is less expensive, has less equipment requirements, but it also is less efficient. Reports are available on the liposome-mediated integration of genes in tobacco (Dekeyser et al., 1990) and wheat (Zhu et al., 1993).

5.8 Microinjection

In this technique DNA is introduced into the nucleus or cytoplasm using a glass microcapillary-injection pipette (Crossway et al., 1986; Morikawa and Yamada, 1985), which requires the use of a micromanipulator. Cells are restrained with a holding pipette and gentle suction while DNA is introduced into the nucleus. Pipettes contain mineral oil that works as a cylinder. Microinjection is mainly used for the conversion of large animal cells and the importance for plant transformation is limited due to the characteristics of plant cell walls. These walls are composed of a thick layer of lignin and cellulose, which is an obstacle for glass microtools. Microinjection of protoplasts could tentatively resolve this limitation; however, this could lead to the release of hydrolases and toxic compounds that could cause swift death of the protoplast. If protoplast viability is not necessary then it is possible to remove vacuoles before microinjection but their loss expressively lowers the ability for division and plant regeneration (Lörz et al., 1981). Different methods of immobilization are required for the microinjection of protoplasts. Rather than using a sucking capillary, protoplasts have been attached by coating the glass with poly-L-lysine or agarose; however, all of these solutions have proven to be harmful as poly-L-lysine can be lethal to some

species and agarose (even a very thin layer) lessens perceptibility in the area of manipulation. Microinjection is currently used for the alteration of large animal cells (e.g., frog egg cells or cells of the mammalian embryos), but it is not routinely used in plants. Disadvantages of the procedure are its slow speed and the requirement for an expensive micromanipulator. However, one of the advantages of microinjection is that it can introduce whole chromosomes into plant cells (Griesbach, 1987; Jones-Villeneuve et al., 1995). It has also become a prevailing tool for studying the cellular functions of plant cells and plastid physiology, (e.g., in tobacco) (Knoblauch et al., 1999). Transgenic plants were recovered at very low frequencies in studies involving tobacco (Schnorf et al., 1991), petunia (Griesbach, 1987), and barley (Holm et al., 2000).

6 Genetically Modified Animals

An animal whose genome has been transformed or modified through recombinant DNA technology is referred to as a GM animal (Bradley et al., 1997). The first GM cattle were reported in 1985 and since then biotechnology developments have allowed researchers to engineer additional GM animals for agricultural and medical purposes. A large number of GM animals are currently undergoing research trials; however, there are a number of animals that are close to reaching the grocery shelf and they will soon be available for marketing. GM cattle have been engineered to improve important traits, such as growth rate, meat quality and quantity, milk production, survival, and disease resistance (Forabosco et al., 2013). Pigs have been modified to grow faster and produce more meat while consuming less feed. Experts have paid special attention to piglets with a focus on improving their health, prolonging their survival, reducing the rate of infectious diseases, and strengthening their immune systems (Watanabe et al., 2012). Sheep have been engineered to improve wool production and resist viral or bacterial infections. The growth rate of chickens and the disease resistance and survival of chicks has also been amplified through engineering (Hu et al., 2013). GM cows and buffaloes with bovine serum encephalopathy (BSE) resistance have also been bred successfully. Moreover, fish have become a target for genetic alterations (i.e., salmon, tilapia, and carp species) with a special focus on increasing the quantity and quality of their meat. GM fish have been sold at pet shops; however, most transgenic animals are still a long way from commercialization due to biosafety and risk assessment issues (Kues and Niemann, 2011).

7 Molecular Advancements in the Development of GM Animals

Most GM animals have been modified for human consumption (i.e., supplied with growth-related genes, such as growth hormone (somatotropin), insulin-like growth factor, and growth hormone releasing factor). Moreover, GM animals with altered enzyme expressions have also been developed. These animals have modified intestinal epithelium that increases the

digestive and absorptive capacity of intestines; for example, animals have been generated with increased phytase activity, which allows the catalysis of organically bound phosphorus (Black, 2007).

Microinjection of foreign DNA into the pronucleus of a mammalian egg or the cytoplasm of a fish egg is a common practice for genetic modification (Dunham and Winn, 2014). Genetic changes can also be performed in mature cells by transferring a nucleus to the egg (Tachibana et al., 2013). Self-directed artificial chromosomes can also be used to accompany these basic techniques for heritable genetic alterations. Artificial chromosomes have several properties in common with natural chromosomes, such as telomeres, centromeres, and satellite DNA; therefore foreign DNA molecules are easily incorporated. The benefit of this transformation system is the reduced risk of uncertainty, which can be inherent during foreign gene insertion. Furthermore, artificial chromosomes replicate as separate bodies and pass through the germline to subsequent generations (Clark and Pazdernik, 2015).

Gene therapy of somatic cells has also been employed for this purpose. Different vectors have been used in different trials; for example, retroviruses become integrated into an animal's genome while adenoviruses remain autonomous (i.e., self-replicating entities) and do not integrate. Gene therapy had been practiced in pigs whereby electroporation was used to target the local expression of plasmids (containing the growth hormone releasing factor gene) to pig muscles. Serum levels of growth hormone and insulin-like growth hormone were claimed to be increased within weeks of gene therapy.

8 Significance of GM Livestock and Fishery

Many GM farm animals have actively contributed to human health through the production of nonfood substances, such as pharmaceuticals, edible vaccines, and organs or tissues (for xenotransplantation or as models of human disease). It has been suggested that GM practices dedicated to economically important traits could possibly fill the spaces created by conventional genetic selection and other non-GM techniques (e.g., embryo transfer and cloning) (Forabosco et al., 2013). A brief summary of the genes transformed into animals, their origin, and their economic significance is provided in Table 1.2.

8.1 Effect of Bt Crops on Nontarget Insects

Bt toxins (i.e., *Cry* proteins) specifically target the epithelial lining of the midgut in susceptible insects. These toxins produce lesions that ultimately result in septicemia in the insect due to the multiplication of enteric bacteria. One of the major aspects of *Bt* crop biosafety is the assessment of *Cry* protein effects in nontarget, beneficial insects (Comas et al., 2014). Experimental studies are conducted by exposing insects to *Bt* crops, such as grains, seeds, or pollens. Approximately 80% of experiments show no significant

Table 1.2: Common examples of transgenic animals, their target traits, involved methodologies, and transgenes.

Species	Target Traits	Transgenic Genes	Methodologies	References
Pig	Growth	<i>Csk1</i>	Transgenesis	Kurome et al. (2006)
	Milk	<i>IGF1</i>	Transgenesis	Monaco et al. (2005)
	Health	Lysozyme	Transgenesis	Tong et al. (2011)
		Unsaturated fatty acids	Transgenesis	Whyte and Prather (2011)
Sheep	Feeding	Phytase	Transgenesis	Whyte and Prather (2011)
	Wool production	<i>IGF1</i>	Transgenesis	Su et al. (1998)
	Health	Galactosyl-transferase and PrP genes	Genetic deletion	Denning et al. (2001)
Goat	Health	Lysozyme	Transgenesis	Scharfen et al. (2007)
	Udder	Stearoyl-CoA desaturase	Transgenesis	Reh et al. (2004)
Chicken	Health	H5N1 resistance	Transgenesis	Lyall et al. (2011)
Cattle	Health	Lysozyme	Transgenesis	Yang et al. (2011)
		BSE	Knockout	Richt et al. (2007)
	Udder	α - and κ -casein	Cisgenesis	Brophy et al. (2003)
		Antimicrobial	Transgenesis	Wall et al. (2005)
Fish	Growth	Follistatin	Cisgenesis	Forabosco et al. (2013)
	Health	Cecropin	Transgenesis	Forabosco et al. (2013)
		Lactoferrin	Transgenesis	
		Lysozyme	Cisgenesis	
	Environmental stress	Antifreeze	Cisgenesis	Ewart et al. (1999)

effects in NTOs and thus support the cultivation of *Bt* crops. *Bt* plants with Cry1Ab and Cry3Bb1 proteins have been commercially cultivated to control pests (i.e., lepidopteran and coleopteran), which are considered to be responsible for significant crop yield losses. To assess the biological safety of *Bt* plants in an ecosystem it is important to remember that the insect of interest should be at high risk of *Bt* exposure or considered as an active part of the food web (Yaqoob et al., 2016).

Losey et al. (1999) suggested that *Bt* maize had a damaging effect on the survival rates of butterfly larvae (*Danaus plexippus*); however, opponents have dismissed these findings as artificially high intensities of *Bt* pollens were used. Subsequent studies using appropriate research conditions found that *Bt* pollens had no effect on nontarget insects. Results and conclusions from several related studies have been summarized in this section (Yaqoob et al., 2016).

8.2 Pollinator Insects

Pollinator insects, such as honeybees and butterflies are supposed to be at high risk from *Bt* maize pollens. Although the overall experimental results prove there is negligible or no effect of *Bt* pollens, some variations do exist between the two risk extremities.

Honeybees (*Apis mellifera* L.) are responsible for more than 80% of the total pollination in natural ecosystems. Babendreier et al. (2007) found minute deposition of *Cry* proteins in hypopharyngeal glands of honeybees but reported that *Bt* toxins do not adversely affect survival rates. Several other studies have since favored *Bt* pollens by documenting their nonlethal effects on the behavior, survival, development, and abundance of honeybees.

Schmidt et al. (2009) performed an experimental study where they fed ladybirds (*Adalia bipunctata*) with *cry1Ab* directly. The authors observed significant lethal effects compared to the control group; however, it was argued that a poor study design might have been the reason for the negative results as later studies confirmed that *cry1Ab* and *cry3Bb1* do not negatively impact ladybirds.

Adult green lace wing (*Chrysoperla carnea*) insects were fed with *Bt* transgenic maize and rice for 1 month but no differences in the fitness and dry weight were observed between *Bt* and non-*Bt* treated groups (Wang et al., 2012). Flower bugs (*Orius insidiosus*) are natural predators that are commonly found in cotton and maize fields. When they were fed with spider mites (supplemented with *Bt*) their early-stage development reduced. Nevertheless, additional experiments confirmed that *Bt* proteins were not fatal for most predatory insects.

8.3 Phytophilous Insects

The butterfly (*Rhopalocera* spp.) is considered to be the most important phytophilous insect. Butterflies suck the sap from plant phloem and play a significant role in food webs and ecosystems, therefore risk assessment studies should be carried out to assess the impact of *Bt* toxins on this species. Numerous studies propose that *Bt* toxins disturb the survival, development, wing size, body weight, and larval behavior of butterflies to a certain extent; however, it is important to remember that although *Bt* pollens can affect the living behavior of butterflies, no deadly effects have been reported in any study. Therefore more critical investigations are required to further assess the impact of *Bt* plants on butterflies (Gustafsson et al., 2015). Other sap-sucking insects, such as aphids (*Aphidoidea* spp.) showed very minute depositions of *Cry1Ab* proteins following exposure to *Bt* maize, although no lethal effects were reported. One reason why *Cry* proteins do not harm aphids or other phytophilous insects could be its reduced translocation into phloem tissues. This would mean that the toxin wouldn't transfer to the aphids when they removed the phloem sap (Guo et al., 2014).

The effects of *Bt* crops on soil macroorganisms (i.e., mites, collembolas, earthworms, and snails) have been studied and no adverse effects were reported in laboratory or field experiments. One experimental approach reported that *Bt* maize negatively affected the growth and egg hatchability of snails (Kramarz et al., 2009); however, the risk was not well demonstrated due to a lack of additional tests. Furthermore, field investigations proposed that crop management protocols, environmental conditions (e.g. rainfall during growing season),

and pesticide use might impact on species diversity and occurrence more strongly than the transgenic crop (Griffiths and Philippot, 2013).

8.4 Effect on Soil Organisms

Earthworms (*Lumbricidae* spp.) are the most vigorous soil organisms and they improve nutrient and water transportation among soil strata. Earthworms directly ingest soil during feeding and therefore it is assumed that *Bt* toxins could influence their fitness when they enter the digestive tract (Icoz and Stotzky, 2008); however, experimental approaches have demonstrated that Cry proteins do not harm the developmental stages or the living characteristics (i.e., growth rate, abundance, reproduction, biomass, and survival rate) of earthworms (Zeilinger et al., 2010).

The nematode (*Caenorhabditis elegans*) is another example of a soil organism that plays a substantial role in nutrient cycling. When the effects of *Bt* crops were assessed in free round worms, it was reported that *C. elegans* were not at risk from Cry proteins and that the diversity was the same between transgenic and control groups (Höss et al., 2011); however, additional experimentations should be performed to further examine the ecological effects of *Bt* crops on soil organisms.

8.5 Effects on Vertebrates

Bt maize is the primary source of feed for poultry hens (*Gallus domesticus*), therefore special attention should be paid to the assessment of biosafety risks in this species. Investigations have assessed the possible adverse effects of *Bt* maize on routine activities and egg qualities of hens and the findings from these studies showed there to be no differences (Rao et al., 2014).

Zhong et al. (2016) fed healthy hens a diet supplemented with *Bt* corn and soybean for 3 months. They established that there were no variations in egg quality, production, or output efficiency between transgenic and control groups. Other parameters, such as internal organ size, *Bt* serum level, behavior, and survival rates were also not affected.

Rats (e.g., Sprague Dawley strain) are important components of the food web and are considered as NTOs. Their 95% genomic and cellular homology to human beings makes them a useful agent for risk assessment studies. As with other research results, no significant effects were observed during a feeding trial in which Cry1Ac protein was continuously administered to rats for 136 days (Shahid et al., 2016). A similar trial was performed whereby transgenic poplar leaves (*Populus cathayana* Rehd) were fed to white rabbits (*Oryctolagus cuniculus*) for 2 months. No significant effects were seen in the weight or health of the rabbits. Histological parameters were not altered and the transgene was not found in the blood or small intestines.

A 2-year study was conducted in Switzerland to assess the impact of GM maize on the fitness and health of dairy cows. Cows were divided into three groups with twelve cows in each group; two groups were fed with *Bt* corn silage, kernels, and cobs while the other group was fed with a non-GM equivalent. The authors concluded that *Bt* corn did not impact milk quality or body condition (Steinke et al., 2010). Similarly, a risk assessment in piglets showed that feeding them with *Bt* maize (MON810 var.) did not have a detrimental effect on gestation, lactation, or developmental (Buzoianu et al., 2013).

Scientists in New York claimed that glyphosate quality in silage resulted in the production of the neurotoxin, botulinum, in ruminants (Ackermann et al., 2015). Therefore glyphosate-resistant crops (i.e., herbicide tolerant) are considered much more effective than conventional crops.

8.6 Effect on Aquatic Organisms

The increased cultivation of GMOs results in enhanced levels of waste being disposed of in water bodies, which may affect aquatic life. Therefore experiments should be designed to estimate the risk that GMOs pose to aquatic life forms, and to assess the direct and indirect effects this may have on human health (as these aquatic animal are used for various purposes including human consumption). Comparatively few studies have been performed to assess the risks to aquatic life than other species (Sissener et al., 2011). In 1996 the first feeding trial was conducted in which a synthetic diet with 45% transgenic soybean was fed to catfish (*Ictalurus punctatus*) for 2.5 months. The authors found that there were no harmful effects on fingerling development, growth, or survival. Similar approaches were performed in rainbow trout (*Oncorhynchus mykiss*) and again, no significant impacts were observed on survival or health (Sissener et al., 2011). However, later studies (Froystad et al., 2008) reported that feeding with *Bt* corn produced increased spleen size with abnormally high lysozyme levels in Atlantic salmon (*Salmo salar*). Nevertheless, one can conclude that GM grains in the diets of fish do not cause drastic harm (Sissener et al., 2010).

8.7 Effect on Human Cells

The effects of transgenic *cp4-epsps* (present in canola) on human intestinal epithelial cells were investigated. In vitro cell cultures were maintained and monitored at regular intervals and the results showed that *cp4-epsps* DNA is rapidly degraded, as multiple fragments were present in the PCR analysis; however, transgenic DNA was present in the cultured cells after internalization (Sharma et al., 2007).

9 Other Unrevealed and Impulsive Effects of GMOs

Different transformational techniques have been employed for the insertion of genes into plant genomes; however, this integration is random and may have unpredictable effects as the insertion could interrupt endogenous plant proteins (Svitashev and Somers, 2001).

For example, 7 years of commercial field cultivation of herbicide tolerant soybean was found to produce RNA rather than active RNA and this was not discovered until several years after the commercialization of Roundup Ready soybean (Rang et al., 2005; Windels et al., 2001). Other irregularities in genetic engineering have also been reported by researchers working on GM insect-resistant maize, who discovered increased levels of unexpected and untested novel proteins in GM crops (De Schrijver and Moens, 2003; Hernandez et al., 2003).

Moreover, hot and dry weather can also pose a serious threat to the yield of GM crops. In these conditions the increased production of lignin leads to stem splitting and yield depletion (Coughlan, 1999). Likewise, a severe reduction in herbicide-tolerant cotton plants has been reported due to the shedding of bolls (Fox, 1997). Moreover, low levels of phytoestrogens have been reported in herbicide-resistant soybeans compared with the conventional variety (Lappe et al., 1999). All of these factors could have serious environmental, animal, and human health effects as environmental factors may also influence transgenesis.

The aforementioned issues have also been reported to correlate with metabolite levels in GM plants. Metabolomic profiling has permitted the identification and quantification of such molecules, and alongside transcriptomic and proteomic analysis has revealed significant differences among GM, non-GM, and control plants (Hoekenga, 2008; Ricroch et al., 2011; Rischer and Oksman-Caldentey, 2006).

These observations demonstrate that the unintended effects of GM crops could be due to mere concerns, a lack of knowledge regarding new technology, or propaganda raised by particular groups, individuals, or organization that intend to delay commercial development of this great technology. GM crops have undoubtedly played a significant role in productivity and these crops have been approved after going through the proper regulatory procedures. So far no significant negative effects have been reported for GM crops (despite the print and electronic media highlighting issues, such as allergies from GM crops) and experimental designs, inaccuracies, reproducibility, and misleading conclusions have been properly addressed by the scientific community (Bakhsh et al., 2015; Nicolai et al., 2014).

10 Risk Assessment

A risk assessment study is compulsory before decisions are made regarding the release and commercial utilization of a GM product. GM risk assessments are currently of significant concern for GM foods and animals earmarked for human consumption. Researchers, consumers, nongovernmental organizations, and critics have proposed that all transgenic varieties of plants and animals should be exposed to long-term animal feeding trials prior to their approval and release in the market (EFSA, 2008). Since the introduction of the first

GMOs, warm debate has continued over the application and release of transgenic technology (Bakhsh et al., 2015). Opponents argue that GM crops with insect-resistance traits could interrupt the natural environment and human health. Others topics that are still being debated include the environmental security, biodiversity disruption, effects of human ingestion of insecticidal proteins and transgenic plant products, adverse effects on NTOs, and economic benefits to farmers in developing countries (Yaqoob et al., 2016).

Researchers have estimated the risks and fears associated with transgenic crops, especially the effect of commercially cultivated plants on the fitness of NTOs. They have used different feeding trials using artificial diets supplemented with GM proteins (e.g., *Bt* protein) under specialized laboratory conditions to study the effects of GM products on the health and behavior of NTOs. However, these investigations were found to be of minor ecological relevance, as controlled laboratory environments cannot exactly simulate what happens when a plant interacts with its natural enemy in the food web. Furthermore, field surveys that focus on an abundance of species are needed to understand the effects of insect resistant crops on ecosystems (Nicolia et al., 2014).

The food safety assessment of GM animals includes the molecular characterization of the inserted gene, its potential unintended effects, and its safety. Allergenicity is another issue that should be addressed in both GM crops and animals and animal products, such as shrimp and cow's milk are known for their intrinsic allergens, (e.g., tropomyosin) (Ladics et al., 2010). Another important point to be considered is the inadequate digestion and intestinal uptake of orally directed proteins and peptides (i.e., hormones) in GM fish. As fish can be turned into feed for other living beings, the potential effects of ectopic protein expression in GM animals warrants further consideration (Kleter and Kuiper, 2002).

The electronic media (including the press) play an important and effective role in disseminating GMO news, both favorable and opposing; however, Domingo and Bordonaba (2011) reported that many consumers are unaware of the virtues and limitations of this transgenic technology. As stated by World Health Organization, national authorities release GM products in the international market after fulfilling the biosafety criteria using animal feeding models, trials, and other relevant parameters (Domingo, 2007; Magaña-Gómez and de la Barca, 2009).

Many scientists report that GM products have the same nutritional value as their conventional source; however, some have documented the presence of histopathological damages (Magaña-Gómez and de la Barca, 2009). Scientists should consider the “substantial equivalence” concept emphasized by the Society of Toxicology (SOT, 2003) when performing toxicological studies to identify the putative differences between GM and non-GM products (Domingo and Bordonaba, 2011). This concept states that if the product under consideration has the same nutritional and compositional properties as the existing product then it must be assumed to be as safe as the original one.

A stalemate has been achieved in the formulation of approved GMO policies. Parties for and against GMOs use every possible opportunity to voice their views. Research groups have published reports describing risk assessment studies of GMOs in critical, informative, and summarizing ways (e.g., a critical series) or published articles on the topic (Domingo, 2000, 2007); however, many opposing reports about GMOs based on results from animal feeding trials have also been questioned and scientifically documented. There is a need to establish a safety assessment system that satisfies the nongovernmental bodies and attracts consumer confidence (Bakhsh et al., 2015).

11 Biosafety Regulations in Pakistan

Pakistan is party to the Cartagena Protocol on Biosafety (CPB). The National Biosafety Centre (NBC) PAK-EPA project was established to accommodate the commitment of the CPB (Yunis, 2015). The main objectives of the NBC are:

- To provide protection against the objectionable effects of GMOs.
- To implement Pakistan Biosafety Rules and the National Biosafety Guidelines as they provide the necessary management and regulatory structure for GMOs.
- To regulate the import, export, production, storage, handling, selling, and release of GMOs.
- To predict the effect of GMOs on plants, animal health, human health, and the environment.

Implementation of these objective is conducted by three types of committees:

1. National Biosafety Committee:

The chairman of this committee is the secretary of the Ministry of Climate Change and the secretary is the director general of Pak-EPA. The main functions of this committee are:

- a. To establish standards and risk assessment protocols.
- b. To consider application(s) for import, export, or commercial release of living modified organisms (LMOs) or reject applications after reviewing the risk assessment in accordance with the biosafety guidelines recommended by the Technical Advisory Committee (TAC).
- c. To prohibit or restrict the import, export, sale, purchase, or trading of any LMOs that cause or are likely to cause risk to public health and safety or the environment.
- d. To develop links with foreign biosafety committees and relevant agencies to guarantee that genetic manipulation practices in Pakistan address international biosafety concerns and observe universal codes of conduct.

The devolution of the subject environment under the 18th amendment has meant that the NBC has faced legal problems; however, the enactment of the Pakistan Biosafety Act of 2015 should lead to these legal issues being resolved.

2. *Technical Advisory Committee:*

The chairperson is the director general of the Pakistan Environmental Protection Agency and the vice chairperson is the director of the National Institute for Biotechnology and Genetic Engineering. The main functions of this committee are:

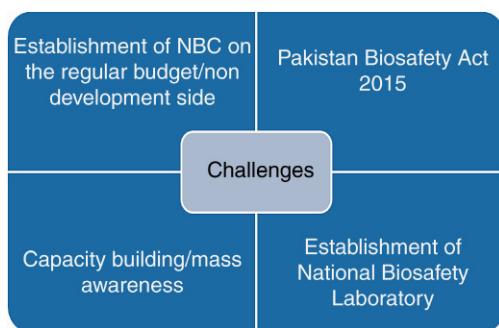
- a. To provide recommended applications for the NBC to allow the release of LMOs, substances, and other concerned products for laboratory work and fieldwork.
- b. To analyze research genetic engineering and recombinant methodologies (and their associated risks) to guide the institution at the international level.
- c. To supervise field applications, experimental field trials, and the release of engineered organisms/products into environment.
- d. To provide data inputs to the NBC for permitted projects under industrial production and if accidents occur during environmental release.
- e. To directly oversee (or oversee through a designated individual) the implementation of terms and conditions set out by the NBC.

3. *Institutional Biosafety Committee:*

- a. Head of the Institution (Chairperson)
- b. Subject Experts and Biosafety Officer (Members)
- c. Social Scientist/Economist (Member)
- d. Representative of Civil Society (Member)

The main functions of this committee are:

1. Help with the exercises of National Biosafety Committee and Technical Advisory Committee.
2. Help researchers undertake hazard evaluation, arrange training programs, and perform experiments in line with biosafety rules.
3. To determine additional safeguards and plan supplementary working to direct the organization in accordance with specific risks and concerns.
4. To regularly (or when required) examine all regulated work occurring within the institution and advise proponents on how to meet biosafety guidelines.
5. To serve as a medium between the NBC and research groups for the flow of data, thoughts, and opinions as is suitable.
6. To ensure the health of laboratory and field personnel in line with their medical records.
7. To coordinate with the NBC and TAC on import, export, manufacture, use, and sale of any GMOs, substances, cells, or products.
8. To collaborate with research teams to prepare plans after the initial assessment of project proposals and risk.



12 Cartagena Protocol on Biosafety

The CPB is an international agreement that has been effective since 2003 as an addition to the Convention on Biological Diversity. It was named in honor of Cartagena, Colombia, where consultations were performed in February 1999. The protocol was finalized on January 29, 2000 in Montreal, Canada, and adopted with the (undisputed) consent of 135 countries.

The CPB seeks to shield biological diversity from the prospective risks posed by GMOs produced using modern biotechnology. The protocol is an international legally binding agreement, which sets out procedures and mechanisms to be followed in the transboundary movement, handling, and transit of LMOs. However, the protocol does not apply to other products of biotechnology (e.g., paper from GM trees) and it is not a cessation on modern biotechnology. In accordance with the provisions of Article 37, the CPB entered into force on September 11, 2003. Up to March 2015 the protocol had 170 parties, including 167 United Nations member states, the State of Palestine, and the European Union. (<http://www.isaaa.org/kc>).

The key objectives of the protocol are:

- To make clear that products from new biotechnologies must be based on precautionary principles.
- To protect biodiversity from the potential risks of LMOs produced by modern biotechnology.
- To balance public health against economic benefits in developing nations.

Key features of the protocol include: ([Cartagena Protocol on Biosafety to the Convention on Biological Diversity, 2000](#)).

1. Advanced informed agreement (AIA): A procedure that must be followed before the first intentional transboundary movement of an LMO into the importing country. This is a notification containing detailed information about the LMO, its regulatory status in

the exporting country, and risk assessments of the LMO provided by the exporter. The importing country must acknowledge this AIA in accordance with the protocol.

2. Biosafety clearing house: It is a website administered by the secretariat of the Convention (<http://bch.biodiv.org>). It was generated to ease the exchange of technical, environmental, scientific, and legal information on, and experience with, LMOs. It also helps parties to implement the protocol.
3. Risk assessment: All decisions on import items must be based on risk assessments that were performed in accordance with scientific protocols based on documented guidelines.
4. Capacity building: Developing countries are provided with the opportunity to use and regulate resources and the capacity to safely use biotechnology and promote the transfer of technology and knowledge within member governments by availing international cooperation through this protocol.
5. Public awareness: Ensuring information is accessible to the public and public discussions are made between member governments.

13 Conclusions

The scientific community has been equipped with knowledge of genetic engineering since the late 20th century. Genetic engineering enables scientists to excise a gene from a specific organism and unite it in another unrelated organism. It has revolutionized breeding methods by breaking hybridization barriers among species or genera and scientists have successfully introduced desired traits against pathogens, weeds, and insect pests in crop plants. Research efforts have attempted to improve crop resistance to biotic and abiotic stresses because significant losses in crop yield are related to these parameters.

14 Future Prospects

Many researchers have been engaged in developing GM animals for the sake of food and feed but none have been commercialized to date. Since the introduction of the first GMO, the application and release of transgenic technology has continued to be a contentious issue.

For example, AquaBounty salmon remains in the FDA approval pipeline despite 15 years of risk assessment studies costing US \$60 million. These studies have confirmed that the allergenic effects of this GM fish are no more than those of Atlantic fish (Bremer et al., 2015). It should be noted that developing countries, such as China and Argentina are spending increasing amounts of money on GM animal research and development and it is expected that many of the GM events will be brought to market in the near future; however, their success or failure will be dependent upon consumer attitudes and acceptance.

By adopting new tools of genetic engineering new varieties have been raised: (1) with improved resistance against fungal and antimicrobial pathogen, (2) with better nutritional

contents, and (3) as bioreactors. Hence these modern tools and technologies should be used in conjunction with conventional breeding techniques. However, more field and farm experiments should be performed and these should be the focus of future studies. The risks associated with GM crops should also be handled carefully (especially in developing countries) and efforts should be directed to gain public confidence. It is highly recommended to establish a trial and safety evaluation procedure to resolve the issues of different nongovernmental organizations who greatly oppose this technology. In conclusion, GM crops are a blessing for humanity as they satisfy the increasing demand of food for increasing populations with limited natural resources under varying climatic irregularities.

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Further Reading

Foods, C.G.M. “Genetically Modified Foods.”

Sugar and Sugar Alcohol Production in Genetically Modified Cyanobacteria

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1 Introduction

In layman's terms the word "sugar" usually refers to a specific carbohydrate, namely sucrose. More generally, "sugars" refers to a multitude of low-molecular weight, soluble carbohydrates, such as glucose and fructose (Fig. 2.1). Sucrose is predominantly used in foods and beverages, but it is also used as a fermentation feedstock for yeasts and other microorganisms in the production of more valuable compounds. Sucrose is produced exclusively from plants, especially sugarcane and sugar beets, with a yearly production of approximately 170 million tons (Anonymous, 2015). Most other sugars employed for practical purposes are produced by chemical, enzymatic, or microbial modification of plant-derived carbohydrates, especially sucrose and starch. One notable exception is lactose, which is of animal origin (whey). "Rare sugars" refer to monosaccharides that are rarely found in nature and are usually expensive to produce (Li et al., 2013; Tang, 2012). These include D-psicose and L-ribose, of which the latter is present in insignificant amounts in nature. "Sugar alcohols" refer to monosaccharides that have been reduced to polyols (Fig. 2.1), some of which have significant commercial interest because they can be used as sugar replacements in foods and beverages (Grembecka, 2015).

Sucrose, glucose, and fructose are used as bulk sweeteners and texturizers in foods (Friedman, 2008). Many alternatives to these sugars are available, and a number of these alternatives are themselves sugars or sugar derivatives, which are characterized by their low calorie content, their low glycemic index, or both (e.g., the polyols sorbitol, xylitol, and erythritol) (Grembecka, 2015). Other sugars including mannitol and mannose are produced commercially in large amounts and have more specialized applications in food and pharmaceutical products. Rare sugars have specialty applications in the food and pharmaceuticals industries (Beerens et al., 2012); for example, L-ribose is used as a building block for anticancer and antiviral drugs.

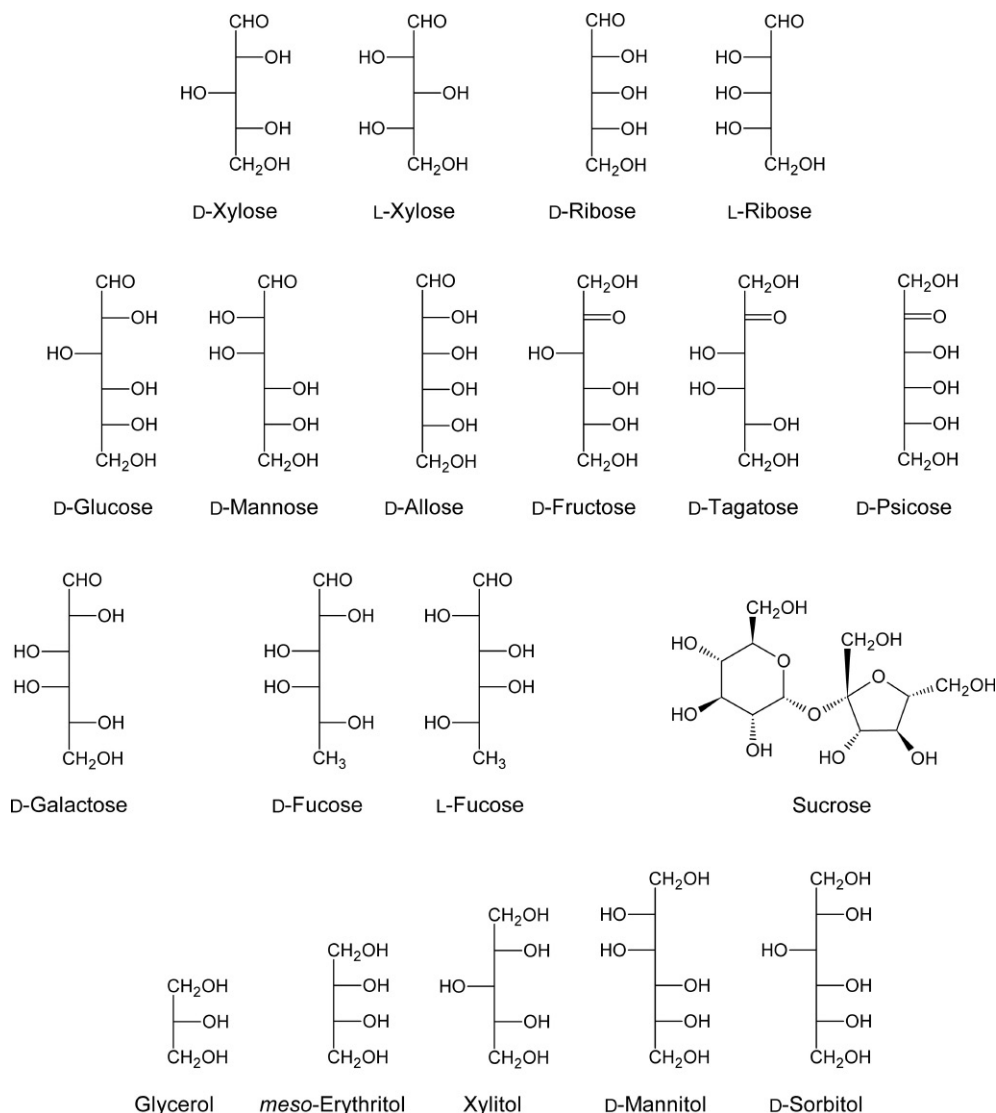


Figure 2.1: Chemical Structures of a Selection of Simple Sugars and Sugar Alcohols.

The majority of sugars used for practical purposes are ultimately derived from photosynthesis. Photosynthesis in plants uses CO₂ to produce sugar phosphates as metabolic intermediates in the CO₂-fixing Calvin cycle. These sugar phosphates are used as the biosynthetic precursors for all other organic compounds in the organism. Sugar phosphates in plants are also converted into storage compounds, such as sucrose and starch. Photosynthesis and the production of sugar compounds occur in a very similar fashion in plants and photosynthetic organisms with the ability to perform plant-like photosynthesis (i.e., macroalgae, microalgae,

and cyanobacteria), although the exact nature of the carbohydrate storage compounds shows some variation (Ball et al., 2011). Polysaccharides that use glucose as a building block may constitute up to 60% of the biomass of some green microalgae and cyanobacteria (Chen et al., 2013; Möllers et al., 2014).

This chapter discusses the use of photosynthetic microorganisms (cyanobacteria) for the production of valuable sugars through genetic modification of their metabolism. A list of candidate sugars and sugar alcohols is shown in Table 2.1. Sucrose, glucose, fructose, and glycerol are naturally produced by cyanobacteria but genetic engineering greatly enhances their production and excretion from cells (Sections 3.2–3.4). Mannitol and erythritol have been produced in cyanobacteria through genetic introduction of novel biosynthetic pathways (Sections 3.5–3.6). If plants and photosynthetic microorganisms are compared for the production of valuable sugars, the latter have the advantage as they grow much faster than plants, and in contrast to plants, photosynthetic microorganisms can be grown in liquid media, into which the produced sugars can be excreted for subsequent retrieval (Section 2.2). In contrast to nonphotosynthetic microorganisms, photosynthetic microorganisms have very simple nutrient requirements and use CO₂ as the sole carbon source. Among the photosynthetic microorganisms, cyanobacteria are good candidates for sugar production for a number of reasons: (1) they exhibit high rates of growth and CO₂ fixation, (2) naturally produce large amounts of carbohydrates, (3) are easy to manipulate genetically, and (4) have a lower cellular complexity than eukaryotic microalgae, making it easier to manipulate desired

Table 2.1: Commercially interesting simple sugars and sugar derivatives.

Names	Formulas	Approximate Values (€ kg ⁻¹) ^a	Applications
D-Psicose	C ₆ H ₁₂ O ₆	> 100,000	Pharmaceuticals
D-Allose	C ₆ H ₁₂ O ₆	70,000	Pharmaceuticals
D-Fucose	C ₆ H ₁₂ O ₅	10,000	Pharmaceuticals
D-Tagatose	C ₆ H ₁₂ O ₆	10,000	Sugar substitute in diet foods
L-Fucose	C ₆ H ₁₂ O ₅	5,000	Pharmaceuticals and cosmetics
L-Ribose	C ₅ H ₁₀ O ₅	1,000	Pharmaceuticals
L-Xylose	C ₅ H ₁₀ O ₅	1,000	Chemical industry
D-Mannose	C ₆ H ₁₂ O ₆	200	Pharmaceuticals and foods
D-Mannitol	C ₆ H ₁₄ O ₆	100	Sugar substitute in diet foods
Meso-erythritol	C ₄ H ₁₀ O ₄	10	Sugar substitute in diet foods
Xylitol	C ₅ H ₁₂ O ₅	10	Sugar substitute in diet foods
D-Xylose	C ₅ H ₁₀ O ₅	10	Sugar substitute in diet foods
D-Fructose	C ₆ H ₁₂ O ₆	5	Bulk sweetener and texturizer
D-Glucose	C ₆ H ₁₂ O ₆	5	Bulk sweetener and texturizer
D-Sorbitol	C ₆ H ₁₄ O ₆	5	Sugar substitute in diet foods
Sucrose	C ₁₂ H ₂₂ O ₁₁	1	Bulk sweetener and texturizer

^aRounded approximate values based on bulk prices of the pure compounds from various anonymous suppliers (2014).

physiological properties including the introduction of novel biosynthetic pathways and excretion of produced sugars.

The cyanobacteria known as spirulina (*Arthrospira* spp.) have been used as food supplements for centuries (Section 4) and much effort is currently devoted to explore the application of various types of cyanobacteria for the sustainable production of biofuels and other products (Angermayr et al., 2015; Hays and Ducat, 2015; Rosgaard et al., 2012; Wijffels et al., 2013). It is possible that the sustainable production of valuable sugars in cyanobacteria may add to the growing list of novel and promising applications for these intriguing organisms.

2 Cyanobacteria

2.1 Description of Cyanobacteria

Cyanobacteria have historically been classified as “blue-green algae” but it is now clear that they are in fact not algae but prokaryotic organisms classified as a separate phylum within bacteria (Cohen and Gurevitz, 2006; Waterbury, 2006). The term “algae” is less well defined but is generally used to describe photosynthetic eukaryotic organisms that do not have the highly developed structures of plants (i.e., roots and leaves) (Graham et al., 2009). Both cyanobacteria and algae have microscopic and macroscopic growth forms, although macroscopic cyanobacteria are much less abundant than their microscopic forms (Mollenhauer et al., 1999). Unlike cyanobacteria, algae do not have a common evolutionary origin (Graham et al., 2009). The term “microalgae” is sometimes incorrectly used to collectively describe eukaryotic and bacterial microorganisms with plant-like photosynthesis, but in this chapter the term “microalgae” is reserved for eukaryotic photosynthetic microorganisms.

Cyanobacteria are unique in that they are the only bacteria that perform plant-like photosynthesis also denoted as oxygenic (O_2 -evolving) photosynthesis. The only other organisms that perform oxygenic photosynthesis are plants and algae. The evolutionary explanation for this similarity is that chloroplasts (the cellular organelles where photosynthesis takes place) in plants and algae are derived from cyanobacteria that established themselves as endosymbionts of a eukaryotic microorganism around 1.5 billion years ago (Yoon et al., 2004). For this reason, cyanobacteria often serve as research models for the study of photosynthesis in plants and algae.

All cyanobacteria require light for growth and their dominant mode of growth is photoautotrophy, although some cyanobacteria assimilate organic compounds to a small extent (Cohen and Gurevitz, 2006; Waterbury, 2006). Some also fix atmospheric nitrogen (N_2) directly to ammonium for growth: a property that is absent in plants and algae. Cyanobacteria are morphologically and developmentally diverse, ranging from unicellular to filamentous and some even exhibit cellular differentiation. They are abundant in all marine and freshwater

environments and some are able to adapt to environmental extremes and survive in very dry environments (i.e., rocks and desert sand), extreme temperature environments (i.e., snow and hot springs), and high salt environments (i.e., salt works and salt marshes). This means that it is possible to isolate producing strains of cyanobacteria that are adapted to a wide range of environmental conditions.

2.2 Large-Scale Production With Cyanobacteria

Approaches to commercial-scale cultivation of cyanobacteria and microalgae are developing rapidly (Chen et al., 2011; Lee, 2001; Slade and Bauen, 2013; Ugwu et al., 2008). Culturing photosynthetic microbes for industrial processes is challenging due to limiting or excess light (Tredici, 2010) and low concentrations of cells and desired products (Angermayr et al., 2015; Lee, 2001; Ugwu et al., 2008). Large-scale commercial cultivation of spirulina (*Arthrospira* spp.), *Dunaliella*, *Chlorella*, and other microalgae for use in health foods or for carotenoid production is carried out in open systems, such as natural or man-made ponds. However, productivity is suboptimal in these systems and the risk of microbial contamination limits their use. Closed systems (i.e., photobioreactors) allow higher productivities and the use of monocultures. There are two dominant types of closed-system cultivation for cyanobacteria and microalgae: tubular and flat-panel photobioreactors, each of which can produce 3–4 g biomass dry weight L⁻¹ day⁻¹ (Lee, 2001). Large-scale production of dried microalgal biomass in a closed-tubular photobioreactor currently costs 5–10 € kg⁻¹ (Norsker et al., 2011; Slade and Bauen, 2013). The costs drop to 2–5 € kg⁻¹ in an open-raceway cultivation system and in the future these costs are anticipated to drop to less than 1 € kg⁻¹ for both systems.

Most cyanobacteria and microalgae production facilities focus on cell recovery (Chen et al., 2011), as the target product is typically either the whole-cell biomass or a valuable compound associated with the cell, such as an oil or a pigment. If the target product is excreted from the cells, such as a low-molecular weight sugar it enters the cell-free fraction of the culture. A number of established bioprocessing techniques can be used to recover target products that are dissolved in the extracellular liquid (Najafpour, 2015). Ultrafiltration and nanofiltration techniques are frequently used for dissolved sugars as they can eliminate particular materials (e.g. cells and lipid globules) and increase the concentration of the target product (Goulas et al., 2003; Yorgun et al., 2008). Chromatography and adsorption can be used to improve purification and crystallization, and spray drying or freeze-drying can be used to produce the final dried sugar product.

Although the techniques to produce and recover soluble sugars from large-scale cyanobacterial cultures are available, a number of important methodological challenges need to be addressed. More specifically, for genetically modified cyanobacteria the challenges lie especially in the genetic stability (Jones, 2014), the low production yield per cell and per

culture volume (Angermayr et al., 2015), and the technological challenges with upscaling cultivation and product recovery (Chen et al., 2011; Slade and Bauen, 2013).

2.3 Synthetic Biology Using Cyanobacteria

Synthetic biology approaches have so far resulted in the production of more than 20 valuable chemicals in cyanobacteria, and this number is rapidly increasing (Angermayr et al., 2015; Hays and Ducat, 2015; Zhou et al., 2016). Cyanobacteria have a simpler cellular structure and genetic makeup than eukaryotic microalgae and thus synthetic biology is currently more advanced in the former. However, genetic manipulation of cyanobacteria for commercial purposes is still in its infancy (Jones, 2014).

Genetic manipulation of cyanobacteria has been described in detail in numerous reviews (Heidorn et al., 2011; Rosgaard et al., 2012; Wilde and Dienst, 2011) and is only briefly mentioned here. Most research is performed using strains that are easily manipulated, including *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, and *Synechococcus* sp. PCC 7942. The premise of synthetic biology is that metabolic pathways that are not present or sufficiently active in an organism can be expressed in a cell through the insertion of novel genes. In cyanobacteria, novel genes are introduced on stable plasmids or integrated into the chromosome by homologous recombination. Selection is typically based on antibiotic resistance genes but several marker-free systems are also available (Begemann et al., 2013; Cheah et al., 2013; Tan et al., 2013; Viola et al., 2014; Wilde and Dienst, 2011). Efforts have also been devoted to understanding and manipulating the metabolic fluxes in cyanobacteria to increase production of the desired product (Angermayr et al., 2015; Zhou et al., 2016).

3 Production of Sugars and Sugar Alcohols

Like most microorganisms, cyanobacteria naturally produce sugars and other compounds as compatible solutes that function in the acclimation to osmotic stress (Hagemann, 2011, 2013). The biosynthetic pathways are reasonably well understood and are shown in Fig. 2.2. Almost all cyanobacteria produce sucrose, and some also produce trehalose, glucosylglycerol, glucosylglycerate, and glycine betaine. These compounds are usually not released to the medium under optimal growth conditions but they may be released from the cells by osmotic shock when transferred into a hypotonic medium. This section describes how genetic manipulation of the metabolic pathways in model cyanobacteria has been used to increase the yield and chemical diversity of sugars and sugar alcohols produced and excreted by these organisms.

3.1 Strategies to Produce Valuable Sugars in Cyanobacteria

The use of enzymes and live microorganisms has dramatically increased the biological toolbox for producing commercially interesting sugars (Li et al., 2013), and some of

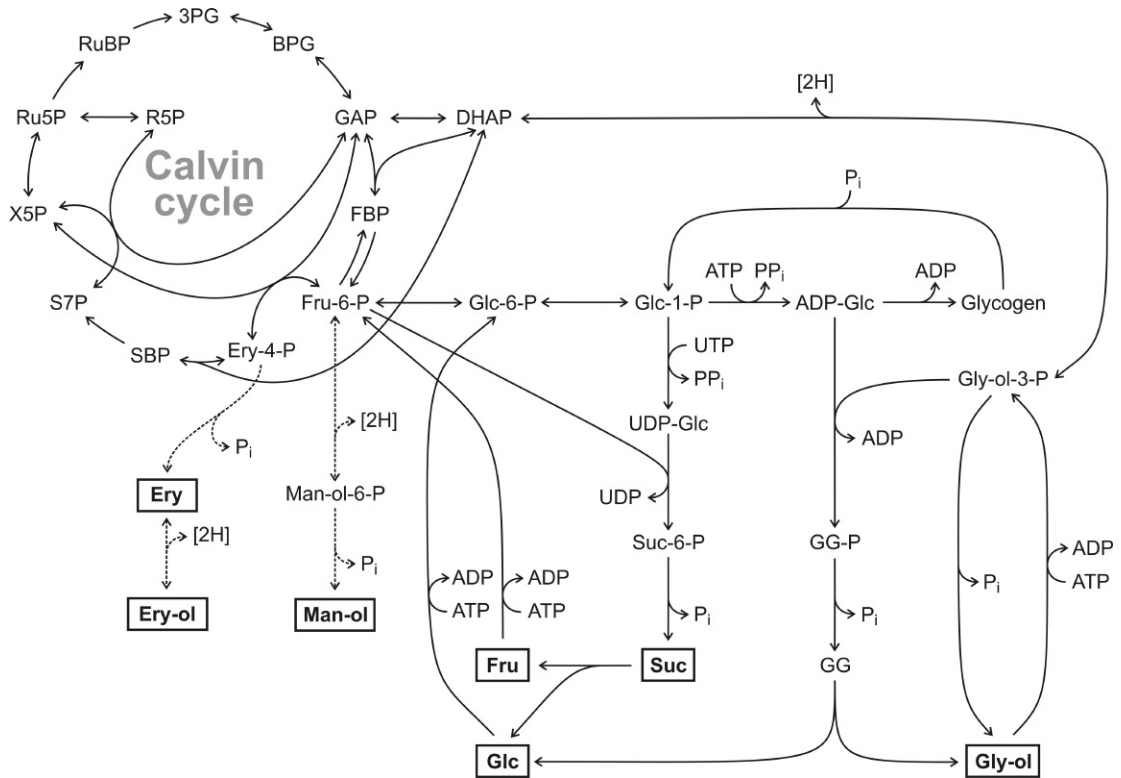


Figure 2.2: Metabolic Pathways Involving Native and Engineered Sugars and Sugar Alcohols in Cyanobacteria.

Not all cyanobacteria have all pathways shown here. Dashed pathways are not known to occur naturally in cyanobacteria. Biotechnologically interesting sugars and sugar alcohols are boxed. Metabolites: *DHAP*, dihydroxyacetone-phosphate; *Ery*, D-erythrose; *Ery-ol*, meso-erythritol; *Fru*, D-fructose; *GAP*, glyceraldehyde-3-phosphate; *GG*, glycosylglycerol; *Glc*, D-glucose; *Gly-ol*, glycerol; *Man-ol*, D-mannitol; *Suc*, sucrose.

these tools may be transferable to cyanobacteria. [Sections 3.2–3.6](#) illustrate how enzymes introduced or enhanced by genetic manipulation can transform abundant sugar phosphates in cyanobacteria into desirable sugars. A number of strategies have been used to increase the productivity and in some cases more than half of the CO₂ fixed by the cyanobacteria is directed into desirable sugar production.

For example, soluble sugar production can be increased by eliminating glycogen synthesis, which otherwise serves as a sink for photosynthesized sugars ([Ducat et al., 2012](#); [Jacobsen and Frigaard, 2014](#); [Xu et al., 2013](#)). Similarly, the synthesis of polyol sugars usually requires reductases that are dependent on either NADH or NADPH. NADPH-dependent enzymes may be preferable because photosynthesis produces NADPH and this molecule is therefore more abundant than NADH in cyanobacteria. The availability of NADH for heterologous biosynthesis in cyanobacteria can be increased through expression of a soluble pyridine

nucleotide transhydrogenase and this has been shown to cause increased lactate production in *Synechococcus* sp. PCC 7942 (Niederholtmeyer et al., 2010). For the efficient production and extracellular secretion of most small sugars it is also important to introduce suitable membrane transporters (Du et al., 2013; Ducat et al., 2012; Jacobsen and Frigaard, 2014; Niederholtmeyer et al., 2010); however, heterologous membrane transporters are not essential for some compounds, such as glycerol and erythritol as these molecules apparently traverse the cytoplasmic membrane without transporters (Savakis et al., 2015; van der Woude et al., 2016). Finally, an inducible genetic system rather than constitutive systems for the synthesis of the desired sugars may be useful as sugar production may inhibit cell growth, thus making the construction, propagation, and genetic stability of mutant cell lines more challenging. The induction mechanism must be inexpensive and nontoxic to be applicable for industrial production.

3.2 Sucrose Production

Synechococcus sp. PCC 7942 is a freshwater cyanobacterium that produces sucrose as the sole compatible solute. Export of sucrose to the growth medium has been obtained by expressing the CscB sucrose:H⁺ symporter from *Escherichia coli* in *Synechococcus* sp. PCC 7942 (Ducat et al., 2012). In this model the expression of *cscB* in *Synechococcus* sp. PCC 7942 was under the control of an IPTG-inducible promoter. Wildtype and CscB-expressing mutants did not produce significant amounts of sucrose when cells were cultivated in standard growth medium (BG11). Sucrose excretion was only observed in the CscB-expressing mutant when NaCl was added and the highest release was observed at 0.15 M NaCl. Moreover, sucrose excretion was not observed when the pH was lower than 7.8, in agreement with CscB being a sucrose:H⁺ symporter. Sucrose production was further investigated by genetic manipulation of carbohydrate metabolism. Glycogen is a major deposit of carbohydrate in cyanobacteria and its synthesis is dependent on ADP-glucose, which itself is formed by ADP-glucose pyrophosphorylase (GlgC; also denoted AGP). In addition, intracellular invertase (InvA) degrades sucrose to glucose and fructose, which are presumably recycled into other cellular components following phosphorylation. Inactivation of *glgC* or *invA* genes caused a slight increase in sucrose excretion (~15%), but the largest increase was observed when both *glgC* and *invA* were inactivated (~25%). Under conditions optimal for sucrose production nearly 80% of the biomass was released as sucrose. The excretion of sucrose in the mutant strains caused an increased photosynthetic CO₂-fixing activity compared to the wildtype, probably as compensation for the biomass loss. The highest productivity obtained was 36 mg sucrose L⁻¹ h⁻¹ with the *cscB*⁺ *invA*⁻ *glgC*⁻ strain in a culture with 1.15 g cell dry weight L⁻¹. This corresponded to a productivity of 0.8 g of sucrose per g of cell dry weight per day. The highest sucrose concentrations obtained without culture toxicity were >3 g L⁻¹. The authors speculated that this high productivity implies that the process may be a viable alternative to sugar synthesis from terrestrial plants (i.e., sugarcane).

Synechocystis sp. PCC 6803 is a freshwater cyanobacterium that produces both sucrose and glucosylglycerol as compatible solutes. Sucrose synthesis is dependent on sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP). The glucose moiety donor for SPS is UDP-glucose, which is synthesized by UDP-glucose pyrophosphorylase (GalU; also denoted UGP). Similarly, glucosylglycerol synthesis is dependent on glucosylglycerol-phosphate synthase (GGPS) and glucosylglycerol-phosphate phosphatase (GGPP). However, in contrast to SPS, the glucose moiety donor for GGPS is ADP-glucose. Overexpression of native *sps* in *Synechocystis* sp. PCC 6803 caused a fourfold increase in the intracellular concentration of sucrose (Du et al., 2013). In contrast, overexpression of native *spp* or *ugp* had little effect on sucrose accumulation, regardless of whether these genes were overexpressed individually or in combination with *sps*. The *ggsS* gene was then inactivated in an attempt to direct carbon flux away from glucosylglycerol synthesis toward sucrose synthesis. This mutation had little effect at the onset of salt stress (0.6 M NaCl); however, after 3 days of growth the *ggsS* mutant strain had accumulated fourfold more intracellular sucrose than the wildtype. Combining all genetic modifications (*sps*⁺ *spp*⁺ *ugp*⁺ *ggsA*⁻) caused an even larger increase in sucrose accumulation. Expression of CscB to facilitate sucrose export did not work well in *Synechocystis* sp. PCC 6803 (as described earlier for *Synechococcus* sp. PCC 7942). At the highest sucrose productivity in the *Synechocystis* sp. PCC 6803 mutant strains investigated, only ~10% of the sucrose was excreted to the extracellular medium. Although the highest sucrose productivity (5–6 mg sucrose L⁻¹ h⁻¹) in *Synechocystis* sp. PCC 6803 (Du et al., 2013) was lower than that for *Synechococcus* sp. PCC 7942 (36 mg sucrose L⁻¹ h⁻¹, Ducat et al., 2012), it is possible that the productivity in the former organism could be increased if a suitable sucrose exporter was identified.

The marine, euryhaline cyanobacterium *Synechococcus* sp. PCC 7002 synthesizes sucrose, glucosylglycerol, and glucosylglycerate as compatible solutes (euryhaline organisms are capable of tolerating a wide range of salt concentrations). Glycogen synthesis was inactivated in this organism through suppression of the two-glycogen synthases genes (*glgA-I* and *glgA-II*) and the effect on sugar production was analyzed (Xu et al., 2013). The wildtype did not excrete significant amounts of soluble sugars under optimal growth conditions; however, the *glgA-I*⁻ *glgA-II*⁻ mutant spontaneously excreted considerable amounts of sucrose and glucosylglycerol to the growth medium. When exposed to a hypotonic or hypertonic shock the mutant strain also liberated more sucrose and glucosylglycerol than the wildtype. A soluble sugar production rate of 14 mg L⁻¹ h⁻¹ was achieved in the *glgA-I*⁻ *glgA-II*⁻ mutant strain. The mechanism of sugar release from *Synechococcus* sp. PCC 7002 is not clear because this organism does not contain known transport proteins that could transfer the sugars across the cytoplasmic membrane. However, the authors proposed that the cells might be able to excrete soluble sugars (and other compounds) through the release of extracellular membrane vesicles derived from the outer membrane. If this is the case, this could be an interesting general mechanism for release of desired metabolites from cyanobacteria.

3.3 Glucose and Fructose Production

Synechococcus sp. PCC 7942 has been engineered to secrete glucose and fructose (Niederholtmeyer et al., 2010). This organism synthesizes sucrose as its sole compatible solute as described in Section 3.2. The *invA* and *glf* genes from *Zymomonas mobilis* were codon optimized for expression in *Synechococcus* and inserted into neutral sites in the chromosome. InvA is an invertase that hydrolyzes sucrose into equimolar amounts of glucose and fructose. The GLF protein facilitates diffusion of glucose and fructose in either direction across the cytoplasmic membrane and the transported sugar is not phosphorylated. When sucrose synthesis was induced in the *Synechococcus invA⁺ glf⁺* mutant strain (through addition of 0.2 M NaCl to the growth medium), glucose and fructose were secreted. It was shown that the simultaneous expression of *invA* and *glf* was necessary for glucose and fructose secretion. The maximum total concentration of glucose and fructose obtained was only 200 μM (equivalent to 36 mg L^{-1}). Interestingly, the glucose levels were significantly lower ($\sim 30 \mu\text{M}$) than the fructose levels ($\sim 160 \mu\text{M}$) and the fructose levels declined as the cell density increased. These observations suggest that glucose and fructose were reassimilated and metabolized by the cyanobacteria. In an attempt to increase intracellular sucrose production the level of the precursor UDP-glucose was increased through expression of the *E. coli* UDP-glucose pyrophosphorylase (GalU). This increased total glucose and fructose production by more than 30% in cells expressing both *invA* and *glf*. This is in contrast to the observation described before (Section 3.2) where overexpression of the native UDP-glucose pyrophosphorylase in *Synechocystis* sp. PCC 6803 did not increase intracellular sucrose accumulation (Du et al., 2013).

3.4 Glycerol Production

Glycerol has multiple uses in the food, chemical, pharmaceutical, and biotechnological industries including as a sweetener, a humectant, a solvent, and a feeding stock for microbial cultures (Christoph et al., 2012). It is commercially produced through the hydrolysis of natural oils and fats from plants and animals.

Glycerol synthesis in yeasts and other organisms occurs through dephosphorylation of glycerol-3-phosphate by one or more glycerol-3-phosphate phosphatases (GPP). Transfer of a GPP from *Saccharomyces cerevisiae* to *Synechocystis* sp. PCC 6803 enabled glycerol production of 11.6 mM (1.1 g L^{-1}) after 17 days, corresponding to $\sim 30\%$ of the fixed CO_2 being rerouted to glycerol (Savakis et al., 2015). A membrane transporter for glycerol export was not employed. Mild salt stress (200 mM NaCl) on the mutant cells increased the extracellular glycerol concentration by more than 20%, presumably because osmotic stress increased the synthesis of the osmoprotectant glucosylglycerol, which in turn led to an increased availability of the intermediate (glycerol-3-phosphate). The highest glycerol concentration obtained was 14.3 mM (1.3 g L^{-1}) after 17 days of culturing and $\sim 50\%$ of

the fixed CO₂ was rerouted to glycerol. The glycerol-producing mutant fully segregated and exhibited stable glycerol production after prolonged serial cultivations, suggesting that the genotype was stable. It is not clear how glycerol was excreted from the cells in this model.

3.5 Erythritol Production

Erythritol is a four-carbon polyol, which is used as a low-calorie sweetener in the food and pharmaceutical industries (Friedman, 2008). Its sweetness level is ~70% that of sucrose and it is safe for diabetics because it does not affect blood glucose levels after oral ingestion. Erythritol and other polyols are not fermented by oral bacteria and therefore they do not contribute to acid production that leads to tooth decay. Erythritol cannot be enzymatically degraded in humans and it is excreted almost exclusively in the urine, therefore it does not have the same laxative effect as other sugar alcohols. Commercial production of erythritol is performed by fermentation using various osmophilic yeasts (i.e., *Moniliella pollinis*) that produce the polyol as a compatible solute (Moon et al., 2010). Yeast and fungal erythritol is synthesized by fermentation of hexoses via erythrose-4-phosphate formed by the pentose phosphate pathway. Phosphatases convert erythrose-4-phosphate to erythrose, which is subsequently reduced to erythritol by erythrose reductase.

The production of erythritol in cyanobacteria was engineered by inserting the pathway known from yeasts in *Synechocystis* sp. PCC 6803 (van der Woude et al., 2016). Erythrose-4-phosphate is an intermediate in the Calvin cycle and therefore should be available in cyanobacteria. Several combinations of phosphatases and reductases from different donor organisms were tested for their ability to convert erythrose-4-phosphate to erythritol. The highest erythritol yield obtained was 2.1 mM (0.256 g L⁻¹) in the cell-free medium of a 28 days old culture. Erythritol did not accumulate intracellularly (<2 mM) even though a membrane transporter for erythritol was not engineered into the cells. Efforts were made to increase the availability of the erythrose-4-phosphate precursor via overexpression of phosphoketolase and transketolase in the cells; however, the effects on erythritol production were minimal. Glycerol production was observed in some strains suggesting that the heterologous phosphatases and reductases were not fully specific for erythrose-4-phosphate and erythrose, respectively.

The relatively low yield of erythritol may be due to a low intracellular concentration of erythrose-4-phosphate. The fluxes of metabolites in the Calvin cycle are very high in cyanobacteria during normal growth in the light (Knoop et al., 2013); however, the steady state concentrations of these metabolites are very different. Concentrations of the sugar phosphates in metabolizing cells are notoriously difficult to determine, although experimental and theoretical investigations have been performed to estimate the concentration of sugar phosphates in the Calvin cycle in chloroplasts (Cruz et al., 2008; Pettersson and Ryde-Pettersson, 1988) and cyanobacteria (Hasunuma et al., 2013). Here, erythrose-4-phosphate

had a consistently low concentration (sometimes below the detection limit) and was among the intermediates with the lowest concentrations (>0.01 mM). In contrast, fructose-6-phosphate had a consistently high concentration (~ 1 mM) and was among the Calvin cycle intermediates with the highest concentrations. Therefore a higher productivity of mannitol derived from fructose-6-phosphate may be achieved compared with erythritol derived from erythrose-4-phosphate. Experimental evidence supports this possibility, as mannitol production in *Synechococcus* sp. PCC 7002 is significantly higher (Section 3.6) than erythritol production in *Synechocystis* sp. PCC 6803.

3.6 Mannitol Production

Mannitol is used in the medical and food industries and is commercially produced by chemical hydrogenation of fructose or extraction from seaweed (Friedman, 2008). Mannitol is found naturally in many plants, algae, fungi, and bacteria and plays roles in energy storage, fermentation, reactive oxygen species scavenging, osmoregulation, and pathogen defense. Mannitol is not produced naturally in cyanobacteria; however, its production in *Synechococcus* sp. PCC 7002 has been achieved through heterologous expression of two codon-optimized genes: NADH-dependent mannitol-1-phosphate dehydrogenase (*mtlD*) from *E. coli* and mannitol-1-phosphatase (*mlp*) from the protozoan chicken parasite *Eimeria tenella* (Jacobsen and Frigaard, 2014). These enzymes convert fructose-6-phosphate (an abundant intermediate of the Calvin cycle) to mannitol. Glycogen synthesis was genetically inactivated (by deleting the *glgA-I* and *glgA-II* genes) to direct carbon flux toward mannitol synthesis and thus increase the mannitol yield. A total mannitol yield equivalent to 10% of cell dry weight was obtained in *Synechococcus* cell cultures synthesizing glycogen, while the yield increased to 32% of cell dry weight in cultures deficient in glycogen synthesis. In both cases $\sim 75\%$ of all mannitol was secreted from the cells by an unknown mechanism. Despite the excretion of mannitol the intracellular concentration of mannitol remained relatively high and was estimated to be ~ 93 mM in the most productive strain. The highest mannitol productivity was obtained in a glycogen-deficient culture after 12 days, which showed a mannitol concentration of 1.1 g L^{-1} and a steady mannitol production rate of $6 \text{ mg L}^{-1} \text{ h}^{-1}$. Mannitol-producing substrains exhibited variations in mannitol productivity such that the productivity was inversely related to the growth rate of the substrain. This variation was attributed to genetic variation among the substrains, which could be caused by a varying degree of chromosomal segregation or the accumulation of secondary mutations.

It is clear that heterologous mannitol production in *Synechococcus* inhibited growth. The wildtype generation time was 6.4 h whereas the strain with the highest mannitol production had a generation time of 28.9 h. In addition, mannitol-producing cells swelled and exhibited intracellular, translucent vesicles of unknown origin, composition, and function (Fig. 2.3). It is possible that these effects could be caused by mannitol biosynthesis interfering with

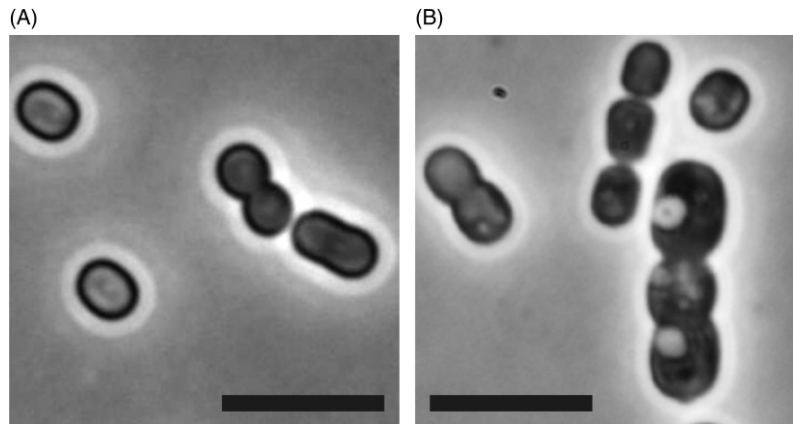


Figure 2.3: Phase-Contrast Light Microscopy Images of *Synechococcus* sp. PCC 7002.
 (A) Wildtype strain. (B) Mutant strain that has been genetically manipulated to synthesize mannitol (Jacobsen and Frigaard, 2014). Scale bars are 10 nm.
 Jacob H. Jacobsen, University of Copenhagen; unpublished.

the concentration and turnover of important metabolites; for example, fructose-6-phosphate is the initial substrate for the engineered mannitol biosynthetic pathway but it is also an intermediate of the Calvin cycle. In addition, mannitol biosynthesis requires additional reducing power (e.g., NADH or NADPH). Finally, the high intracellular concentration of mannitol may be detrimental to the cells by causing a high intracellular osmotic pressure. If this is the case the introduction of a cytoplasmic transporter for the secretion of mannitol may alleviate the growth inhibition. A genetic system that allows the controlled induction of mannitol synthesis would also be beneficial, as this will allow maintenance and propagation of the culture in the absence of mannitol production; however, both of these possibilities remain to be tested.

4 Human Health Concerns About Cyanobacteria

Some cyanobacteria produce compounds that are toxic to humans and animals (Dittmann et al., 2013; Jakubowska and Szelag-Wasielewska, 2015; Rastogi et al., 2015); however, due to the large diversity of cyanobacteria this is by no means all encompassing. Cyanobacteria (i.e., *Arthrospira platensis*, commonly known as spirulina) that are used in the production of nutrient supplements and food colorings for human consumption are labeled as “generally regarded as safe” (GRAS) (Belay, 2007). Other cyanobacteria, such as *Aphanizomenon flos-aquae*, *Aphanothece sacrum*, *Nostochopsis* spp., and various *Nostoc* species including *Nostoc commune*, *Nostoc verrucosum*, and *Nostoc microscopium* have been consumed by humans for centuries (Carmichael et al., 2000; Emralino and Rodulfo, 1987; Han et al., 2013; Motham et al., 2014; Oku et al., 2014). The cyanobacterial model strains that are most

frequently used for synthetic biology studies (*Synechococcus* sp. PCC 7002, *Synechococcus* sp. PCC 7942, and *Synechocystis* sp. PCC 6803; Sections 3.2–3.6) are not known to produce toxins.

One approach for testing the toxicity of cyanobacteria in animals has been to feed whole cells or cell extracts of various cyanobacterial strains to mosquito larvae and record the development of the larvae into normal adults (Kiviranta et al., 1993; Stevens et al., 1994). Larvae toxicity was found to be associated with the presence of hepatotoxins and neurotoxins in the cyanobacterial samples. Many strains of cyanobacteria (including the aforementioned strains PCC 6803, PCC 7002, and PCC 7942) were nontoxic to mosquito larvae.

Toxins from cyanobacteria (cyanotoxins) are divided into three main chemical groups: cyclic peptides, alkaloids, and lipopolysaccharides (LPS). These cyanotoxins can also be biologically classified into five functional groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins. Many reviews focus on these toxins and the specific cyanobacteria that produce them (Dittmann et al., 2013; Jakubowska and Szelag-Wasielewska, 2015; Rastogi et al., 2015).

LPS are the major components of the outer membrane of Gram-negative bacteria including cyanobacteria. LPS from many bacteria elicit a strong immune response in humans and cause allergies, respiratory diseases, and skin diseases. LPS from cyanobacteria also exhibit toxicity in humans but typically much less than LPS from enteric bacteria (e.g., *E. coli*), and LPS from some cyanobacteria (e.g., *Anabaena flos-aquae*) show no toxicity at all.

Reports have suggested that the neurotoxin β -N-methylamino-L-alanine (BMAA) is produced by the vast majority of cyanobacteria (Cox et al., 2005); however, recent work suggests that this assumption is erroneous and only a very limited number of cyanobacteria (if any) produce measurable amounts of BMAA (Faassen, 2014; Li et al., 2012; McCarron et al., 2014). BMAA has not been detected in spirulina (*Arthrospira* spp.) (McCarron et al., 2014).

It should be noted that the cultivation of harmless cyanobacteria for human use is at risk of contamination by hazardous cyanobacteria, and this is especially true in open cultivation systems, such as ponds (Section 2.2). For example, cyanotoxins have been identified in spirulina products, but this may be attributed to contamination with toxic cyanobacteria and not to *Arthrospira* spp. (Belay, 2007).

5 Conclusions

Cyanobacteria represent a novel approach for producing valuable sugars and sugar alcohols using synthetic biology. Cyanobacteria producing strains that are nonhazardous to humans are available and more could be identified if required (Section 4). Moreover, the methods for cultivation and recovery of the dissolved target products are also available or are being

developed (Section 2.2). However, it is necessary to develop methods to increase productivity and the genetic and phenotypic stability of the production organisms (Section 3). It is also necessary to identify more biosynthetic enzymes and membrane transport enzymes to allow a greater diversity of target products to be produced (Li et al., 2013). Low molecular weight, water-soluble sugars cannot accumulate intracellularly in the production organism without serious impacts on cellular physiology, therefore the sugars need to be excreted from the cells, preferably by means of specific membrane transporters. The approach to produce valuable sugars and sugar alcohols in cyanobacteria as presented here will need to be compared to alternative approaches, such as chemical synthesis, in vitro enzymatic conversions, and substrate conversion using live chemoheterotrophic microorganisms to evaluate if it is of practical interest.

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Genetically Modified Plants as Sustainable and Economic Sources for RUTFs

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1 Introduction

1.1 Food and Human Nutrition

Food is broadly used to refer to any substance that is consumed by organisms (including humans) to provide energy and the basic building molecules of life (e.g., proteins, minerals, vitamins, complex biocompounds, lipids, and carbohydrates). Food is necessary for any organism to grow and develop. Plants are the main source of food as they are at the bottom of the food pyramid. The most important food sources (known as staple foods) are plant products, such as cereal grains with wheat and rice being essential for human nutrition and corn as a livestock feed. Many domesticated animals and birds are the second most important source of food for humans, either directly or by their by-products. Humans began to domesticate and raise wild animals for food, for their various by-products, or for their work capacity. Meat, milk, eggs, and dairy products are examples of animal-based foods. Some foods, however, are not obtained from animals or plants and although these foods are not considered as staple foods they can add flavor, color, and additional nutritional values to existing food forms, to make different forms of the same food, or to delicately change the nature of some foods into new ones. The most famous examples in this category are edible fungi (e.g., *Agaricus bisporus* and *Lentinula edodes*), bacteria (e.g., *Lactobacillus* spp.) used in the food processing industry, and blue-green algae (e.g., *Spirulina* sp.). In addition to organic or live food sources, there are other inorganic and earthy substances that can be used as food-preserving additives or to chemically alter the characteristics of foods. Culinary salt (NaCl) and baking soda fall within this category.

1.2 Malnutrition

The human body requires sufficient amounts of energy and various nutrients to enable proper growth and development. The nutritional need varies with age and is directly linked to genetics, physiology, gender, and the environment in which the person lives. If the body receives less or more than is needed, to the point that health is adversely affected, it is interpreted as bad nutrition or malnutrition. By definition, malnutrition is “a state in which the physical function of an individual is impaired to the point where he or she can no longer maintain adequate performance process, such as growth, pregnancy, lactation, physical work, and resisting and recovering from disease” (WFP, 2004). Although under nutrition (or its more severe form, hunger) is often highlighted, both under- and overnutrition are equally as bad. The global prevalence of malnutrition is devastatingly high, with approximately 1 billion people adversely affected by undernutrition with another 1 billion suffering from overnutrition (i.e., obesity). The short- and long-term consequences of malnutrition could have major impacts on social communities and countries. In the short term, malnutrition often weakens the immune system and puts individuals at increased risk of disease and infection, which may ultimately end in death if not treated in time (Walton and Allen, 2011). It is estimated that two-thirds of the global burden of disease will be due to chronic noncommunicable illnesses by 2020, most of which will be related to the human diet (Chopra et al., 2002). In the long term, malnutrition can reduce the quality of life by causing various physical and mental illnesses including mental developmental problems, educational underachievement, and a reduced ability to work. Malnutrition would also lead to long-term nationwide problems for families and societies. As affected people have a reduced ability to work, this is followed by a cycle of household hunger and poverty, as there are fewer incomes in the household. This could hinder the economical and welfare development of a country (FAO, 2012c; Walton and Allen, 2011).

Malnutrition can be broadly used to refer to hunger in underdeveloped countries, obesity in developed nations, or nutrient deficiencies (i.e., iron or zinc) in many developing countries; however, it should be noted that global distribution of malnutrition is no longer straightforward. The World Health Organization (WHO) stated that “in the poorest countries, even though infectious diseases and undernutrition dominate their current disease burden, the major risk factors for chronic diseases are spreading. The prevalence of overweight and obesity is increasing in developing countries, and even in low-income groups in richer countries” (WHO, 2004). A report published by the Food and Agriculture Organization (FAO) of the United Nations in 2012 clearly supported this definition of the problem and revealed that although the number of people suffering from malnutrition around the world had decreased between the years of 1990 and 2012, the number of malnourished people in some less-developed parts of the world, such as sub-Saharan Africa had increased from 17% to 27% during this time period (FAO, 2012b). The degree to which a person is suffering from malnutrition and consequently requires attention is diverse and various factors can influence

Table 3.1: The World Food Program's classification of malnutrition on the basis of Z-score values.

Classification	Z-Score Values
Adequate	$-2 < Z\text{-score} < 2$
Moderately malnourished	$-3 < Z\text{-score} < -2$
Severely malnourished	$Z\text{-score} < -3$

this. All age groups can be affected by malnutrition, but the most nutritionally vulnerable groups are infants, young children, and pregnant or lactating women, because of their high physiological nutritional requirements (Lartey, 2008).

Malnutrition can be divided into two general types based on its cause: macro- or micronutrient deficiency (Müller and Krawinkel, 2005). The World Food Program's criterion uses a Z-score value to broadly classify malnutrition as adequate, moderately malnourished, or severely malnourished (Table 3.1) (WHO-UNICEF, 2006). The severity of malnutrition is also split into two levels: acute and chronic (Collins et al., 2006). Macronutrient malnutrition occurs when a diet is frequently deficient in carbohydrates and proteins, which results in protein-energy malnutrition (Müller and Krawinkel, 2005). Moderate acute malnutrition is associated with a weight-for-height Z-score (WHZ) between -2 and -3 while severe acute malnutrition (SAM) is defined as a WHZ of < -3 . Approximately 33 million children under 5 years of age were affected by moderate acute malnutrition in 2012. (De Onis et al., 2012; WHO, 2013). Chronic malnutrition (stunting) is expressed by height-for-age measurements (Black et al., 2008). Micronutrients deficiencies are more common among developing nations (Mason et al., 2005) despite the food supply being adequate to meet the bodies energy requirements. They are usually caused by little variation in the daily diet. This type of malnutrition does not exhibit the known symptoms of macronutrients deficiencies and it is often referred to as the "hidden hunger." The three micronutrient deficiencies that are the most common and life-threatening are vitamin A, iron, and iodine deficiencies (Ramakrishnan and Huffman, 2008). Vitamin A deficiency may lead to poor vision and an impaired immune system (West and Darnton-Hill, 2008), iron deficiency is the most common cause of anemia (Gupta et al., 2016), and iodine deficiency during pregnancy is known to cause irreversible brain damage in infants (de Escobar et al., 2007; Delange, 2001). The effects of these three nutrients alone might have a negative effect on an individual and the society.

Malnutrition in most parts of the world is usually associated with, or caused by, a series of interrelated social, economic, political, geographical, and cultural conditions. Poverty can limit an individual's access to proper and sufficient living conditions, such as health services, food, and education, which ultimately leads to vulnerability to malnutrition (Kaseje et al., 2005). Poverty is a challenging and common problem in many underdeveloped countries. Hampshire et al. (2009) reported that 63% of the 12.4 million people in Niger lived on less than US \$1 per day. Children from poor families are usually more prone to infectious

diseases, receive less nutritious foods, are exposed to more unhealthy environments, and have reduced access to health services compared to children from wealthier families (Bryce et al., 2008). Food insecurity is another cause of widespread malnutrition. Problems occur when people do not have physical and economic access to a safe, nutritious, and sufficient food supply (Black et al., 2008). The food insecurity is common, even among developing countries, and by definition it could be classified as chronic, transitory, and seasonal. Chronic food insecurity is the inability of a person to obtain the minimum nutrients needed over a long period of time, transitory food insecurity refers to a sudden decline in access to food over a short time period, while seasonal food insecurity is caused by changes in the local climate and agricultural activities that affect the local or regional food production and supply (FAO, 2012a). The civil unrest and wars are well-known causes of malnutrition (Bryce et al., 2008) and O'Hare and Southall (2007) showed that there was a link between civil conflicts and the extent of malnutrition and infant mortality in sub Saharan Africa.

1.3 Global Demand for More Value in Foods: A Health Need or a Social Trend?

The growing need for food is a well-established fact; however, the fact that more food does not necessarily mean better nutrition is gaining attention. The quality of daily food in some societies, and the enhanced quantity of staple foods in other societies, used to be a matter of discrimination among developed and underdeveloped nations as food quality was a status symbol. However, this trend is rapidly finding its way into developing and underdeveloped nations where food scarcity is a more prominent issue. Therefore, although the need for added value staple foods used to be a trend in some societies, it is now a need for most of the global population. There are a few reasons for this trend. The global population is aging and life expectancy continues to rise; interestingly, this is a two-way phenomenon. People live longer because they receive more value in their diet and have access to better healthcare, which in turn leads to an increased demand for more nutrition and benefits in the food they take. Another reason is public education and people are now more nutrition savvy than ever before. They can optimize the health-promoting capabilities of their diet through supplementation and by consuming foods that have been formulated or fortified to include health-promoting factors. In other words, people do not like to eat a lot but they like to eat foods of higher value. Consequently, functional foods have emerged to meet this demand. Politics and economics play import roles in the promotion of functional foods. The production of staple foods has become more expensive in various parts of the world, which is due to the various negative effects of global warming and climate change, such as water scarcity, agricultural water decline, and increased soil salinity, or anthropogenic activities, such as shrinking arable lands due to the expansion of cities. Therefore, it might be more economical to produce reduced volumes of food but with higher values in terms of energy, nutrition, and health benefits. The demand for added value foods is no longer solely a trend for developed nations or luxurious lives, but it has become a need for almost all societies.

The proper and timely response to human nutritional and energy demands is critical to prevent or alleviate the short- and long-term effects of malnutrition. The first reaction to acute malnutrition has traditionally been hospitalization and treatment with energy- and nutrient-rich liquid diets containing ingredients, such as milk powder, vegetable oil, and sugars fortified with vitamins and minerals (Ciliberto et al., 2005; Manary and Sandige, 2008). Such approaches have generally been successful in helping patients recover from acute life-risking malnutrition if the treatment is given in time. However, the treatment for each patient may vary based on the severity of the malnutrition and the availability and access to resources and medical facilities. More importantly, responding to outbreaks of severe malnutrition requires the allocation of huge amounts of money and logistics, which are not economically and logically feasible in regions where malnutrition is a common problem. Therefore, feasible and sustainable solutions for daily nonemergency situations to prevent or correct common malnutrition could be more efficient. To this end, the WHO recommended a general plan to treat moderate acute malnutrition using daily food rations, which provide the energy and other nutrients missing from the diet and are consumed in addition to a person's regular daily diet (Walton and Allen, 2011). These types of energy-rich foods are known as supplementary or complementary foods (Manary and Sandige, 2008) and mostly include plant based foods, such as cereals and legumes fortified with micronutrients and mixed with a blend of corn and soy flour (Patel et al., 2005).

1.4 Functional Foods

Functional foods are typically nutritional- and energy-enriched meals that serve a specific purpose or target a special group in a society. Hasler (2002) defined functional foods as “whole, fortified, enriched, or enhanced foods that provide health benefits beyond the provision of essential nutrients (e.g., vitamins and minerals), when they are consumed at efficacious levels as part of a varied diet on a regular basis.” Therapeutic foods are famous functional foods and generally refer to any type of food with a specific medicinal or healing effect in both preventative and correctional programs. They are different from medicines as they are added to energy-rich foods for daily consumption. Their consumption is also safe for healthy individuals even if they do not fall within the target population for the food. Supplementary and complementary foods are also functional foods that target nutrient deficiencies and other types of malnutrition. Supplementary foods are formulated to supply all essential nutrients (especially micronutrients) in one compact recipe. Complementary foods tend to be bulkier than supplemental foods and they usually supply a smaller amount of nutrients in a single meal. Complementary foods may contain selective microelements (i.e., zinc or iron), proteins and amino acids, or micronutrients with/without enzymes. Functional foods may be commercially produced or locally made and they are available in various edible forms including solid (e.g., powders, blends, biscuits, or candies), semisolid (creams, pastes), and liquid forms.

2 Ready-To-Use Therapeutic Foods

Despite the many advantages of supplementary, complementary, and therapeutic foods, there are some limitations for their long-term use and applications where they are prepared in liquid forms or require precooking. Therefore, new preparation techniques are needed to increase their shelf life, ease of consumption, and overall nutritional value to consumers, particularly younger people in underdeveloped nations. Such foods are commonly referred to as ready-to-use therapeutic foods (RUTFs) (Briend and Collins, 2010). RUTFs can be defined as “energy-dense, micronutrient-enriched foods, which are soft or crushable or drinkable in a way that can be directly consumed by people without any prior cooking.” These functional therapeutic meals can be prepared on any scale, from homemade recipes with locally available ingredients in remote villages to highly technical formulations in food factories; however, to conform to the WHO requirements, RUTFs should include a specific amount of readily available energy and a variety of micronutrients and vitamins. Table 3.2 summarizes the RUTFs energy and nutritional values that have been suggested by the WHO (1999, 2013). In addition to fulfilling certain energy and nutritional criteria, Dubey and Bhattacharya (2011) mentioned that RUTFs should also be simple to store and deliver, easy to use, stable under varied climates and temperatures, fast acting, resistant to bacterial growth, safe to store in ambient tropical conditions without spoiling for many months (Briend, 2002), nonaddictive, free from poisonous substances (i.e., heavy metals or agrochemicals), economically affordable, culturally acceptable, easy to administer, and require little or no preparation before use. These extra characteristics are what make RUTFs different from other therapeutic or functional foods. The WHO recommends that the treatment of SAM in children should contain moderate levels of positive (nonmetabolizable) bases to decrease the risk of metabolic acidosis (WHO, 2013).

RUTFs typically contain a few powdered ingredients, such as milk, sugar, vegetable oil, minerals, vitamins, and peanut butter as an embedding paste. The production of RUTFs is not a complicated process and it largely consists of grinding and mixing the ingredients in proper proportions and packaging the final product (Manary, 2006). The following steps can be used for making a typical RUTF: (1) all raw ingredients are dried and grinded into powder form; (2) the powdered ingredients including ground peanuts, skimmed milk powder, sugar, minerals, and vitamins are blended with vegetable oil containing less than 0.1% emulsifiers and some antioxidants; (3) the resulting paste is homogenized to reduce its particle size ($<200\text{ }\mu\text{m}$), and (4) the paste is packaged (Beesabathuni and Natchu, 2010). A major issue in the production of RUTFs is the quality control and making sure that the mixture is not contaminated with any heavy metals, pesticides, or agrochemicals. It is especially important to ensure that aflatoxin contamination is tested when using peanuts in these types of foods (Egal et al., 2005). Lipid oxidation activity, fat and protein content, total moisture content and water activity, and vitamin and mineral concentrations should also be checked in these foods.

Table 3.2: Recommended nutritional composition of RUTFs by the WHO.

Nutritional Content	Amounts
Moisture content	2.5% maximum
Energy	520–550 kcal/100 g
Proteins	10%–12% total energy
Lipids	45%–60% total energy
Sodium	290 mg/100 g maximum
Potassium	1110–1400 mg/100 g
Calcium	300–600 mg/100 g
Phosphorus (excluding phytates)	300–600 mg/100 g
Magnesium	80–140 mg/100 g
Iron	10–14 mg/100 g
Zinc	11–14 mg/100 g
Copper	1.4–1.8 mg/100 g
Selenium	20–40 µg
Iodine	70–140 µg/100 g
Vitamin A	0.8–1.1 µg/100 g
Vitamin D	15–20 µg/100 g
Vitamin E	20 mg/100 g minimum
Vitamin K	15–30 µg/100 g
Vitamin B1	0.5 mg/100 g minimum
Vitamin B2	1.6 mg/100 g minimum
Vitamin C	50 mg/100 g minimum
Vitamin B6	0.6 mg/100 g minimum
Vitamin B12	1.6 µg/100 g minimum
Folic acid	200 µg/100 g minimum
Niacin	5 mg/100 g minimum
Pantothenic acid	3 mg/100 g minimum
Biotin	60 µg/100 g minimum
<i>n</i> -6 fatty acids	3%–10% of total energy
<i>n</i> -3 fatty acids	0.3%–2.5% of total energy

The provision of globally accepted and regulated standards is required to cover the formulation, preparation, trading, and administration of RUTFs. This is even more critical for those products that are intended to act as medical foods and where their consumers are already suffering from various nutrient-related weaknesses and are highly vulnerable. Therefore, the Codex Alimentarius commission was established by the FAO and the WHO in 1963 to act as a world authority for international food and to develop and regulate internationally-accepted guidelines for the food industry ([Codex Alimentarius, 2005](#); [FAO, 2016](#)). The purpose of the commission was to propose, evaluate, and regulate international food product standards ranging from formulation programming to supply chains and trading. The standards proposed could offer the possibilities to improve the quality and accessibility of RUTFs to the global community. The clear definition for the product type, composition, packaging, labeling specifications, and its intended purpose and regulations

may make it easier to trade newly-developed foods, which would facilitate its global trade and support the supply-chain security. In addition, it serves as a preemptive checkpoint to protect the end users by ensuring the quality and safety of the products before it reaches them; however, it is important that such standards and regulations do not become an obstacle for developing innovative foods, which are specifically designed to serve different nutrition demands in different societies (Schweitzer, 2016). Therefore, the codex guidelines and codes of practice for various food products are routinely updated to contribute to the safety, quality, and fairness of the international food trade (FAO, 2016).

3 Perspectives for Alternative Sources

The high expenses of preparing RUTFs influence their sustainability and the effectiveness of nutrition development programs in many countries, especially developing countries. Some of the main ingredients in RUTFs are also not commonly available in most developing countries, which makes them expensive to produce and difficult to access. In 2006 the WHO estimated that 70% of RUTF production costs were attributable to their main nutritional additives (i.e., milk powder) (WHO-UNICEF, 2006); however, almost a decade later the cost of RUTFs is still one of the main obstacles for scaling up community-based management of acute malnutrition (CMAM) among developing societies (Dibari et al., 2012). Therefore, the identification and application of cheaper alternative RUTF ingredients, either within the context of current functional food recipes or new innovative recipes, is a global public health priority. One solution could be to investigate the potential of locally produced crops or agricultural by-products to significantly reduce the costs of base ingredients. The indigenous or locally produced RUTFs have some advantages over imported commercial RUTFs; for example, they may not require such long shelf lives as they would be consumed locally soon after production. They may also promote local agricultural programs and activities to support the demand for their ingredients, which may in turn contribute to the economic status of local farming families. The therapeutic foods that are locally produced could be readily distributed by local communities and may be more culturally welcomed by the society (Dubey and Bhattacharya, 2011). This strategy may also support the sustainable and economical efficacy of nutritional developing programs, and the scientific community had already begun looking into it.

4 Crop Plants

Plants are known to be rich in vitamins and various nutritional elements (especially micronutrients) that contribute to a healthy diet (Gupta and Bains, 2006); however, a single vegetable might not provide all the nutritional requirements and therefore a diversified diet is needed to meet daily micronutrient requirements. Uusiku et al. (2010) reviewed the

literature regarding the nutritional value of African leafy vegetables that are consumed in sub Saharan Africa compared with exotic imported vegetables. They evaluated the potential impacts of the endemic plant sources in daily diets with respect to processing methods and the presence of antinutritional factors (ANFs), which might adversely affect the nutritional value of such foods. Many of the wild vegetative species in the area were found to contain high levels of micronutrients and vitamins, such as cassava (*Manihot esculenta*) (1970 mg retinol equivalents/100 g edible portion and vitamin C at 311 mg/100 g), *Chenopodium album* (iron at 6 mg/100 g, zinc at 18.5 mg/100 g, calcium at 226 mg/100 g, and magnesium at 211 mg/100 g), *Arachis hypogea* and *Bidens pilosa* (with high levels of dietary fiber), and *Nasturtium aquatica*, *Urtica dioica*, and *Xanthosoma mafaffa*, which were rich in antioxidant agents. They concluded that the endemic vegetable-based foods could account for a considerable proportion of daily nutritional needs, in some cases even more than nutritional imported exotic vegetables, such as spinach or cabbages; especially in individuals with marginal nutritional status. Bahwere et al. (2009) described a pilot program to evaluate the effectiveness and acceptability of a model RUTF made out of local crops for nutritional support of sick HIV-positive adults in Malawi. In this crop, the commonly used milk powder ingredient had been removed from the formula, and the food consisted of 20% vegetable, 15% sugar, 22.4% sesame seeds, 36% chickpeas, 5% maize, and 1.6% vitamins and minerals. Their results indicated that the locally produced RUTF was acceptable to patients and was associated with a rapid weight gain and enhanced physical activity performance. The intervention is likely to be more cost effective than nutritional support using food-aid commodities. Nabuuma et al. (2013) deployed the Cost Formulation System computer software (The Concept-4, Creative Formulation Concepts LLC, Annapolis, MD, USA) to devise the best feasible plant-based RUTF formula that could meet the nutrient composition of F100 (WHO, 1999). They evaluated ten locally grown crops in Uganda including bananas, peanuts, cowpeas, orange-fleshed sweet potatoes, corn, sorghum, sesame, beans, millet, and grain amaranth according to their average nutrient compositions (Gebhardt et al., 2008), their mean price, amino acid composition, and energy content. The three predicted formulations with the nutrient composition closest to the target F100 protocol were then evaluated. They showed that plant-based therapeutic foods received consumer acceptability of 65%, which showed the economic feasibility of such therapeutic foods in the management of malnourished underdeveloped societies. Bechman et al. (2015) formulated in situ food supplement formulas using formulation computer software (CFC4-S2; Creative Formulation Concepts, LLC, Annapolis, MD) aimed at developing low-cost, plant-based RUTFs for the treatment of malnourished lactating and pregnant women in Mali in West Africa. They investigated the possibility of using the agricultural foods, which were commonly consumed, and had the highest production rates in Mali (FAO Production Stat, 2013). Crop plants that were considered as potential ingredients for the RUTF formula included peanuts, sorghum, rice, cowpeas, yams, fonio, millet, sugar, corn, cassava, sesame, and barley. They also

enriched the formula with plant enzymes from rice and barley, which were fermented with *Aspergillus oryzae*. The resulting fermented product, known as koji, usually contained a few natural enzymes, such as glucoamylases, α - and β -amylase, α -glucosidase, and neutral and acid proteases (Chou, 1995). The components of the six selected formulations were milled, hydrolyzed with koji α -amylase, and heated at 121°C for 15 min. The chemical contents (i.e., protein, fat, ash, fiber, carbohydrates, amino acids, and energy of dehydrated products) of the six selected formulations were determined and then compared with the software-predicted values. Aside from a few differences, the overall efficacy of the formulas were acceptable and the production costs of the RUTFs were meaningfully lower than imported commercial RUTFs, even with increased ingredient prices in Mali from 2004 to 2009. The authors insisted on providing all possible ingredients in the RUTF formula from plant sources without compromising on the price or the final quality of the products. More recently, Bahwere et al. (2016) compared the efficacy of a soya–maize–sorghum RUTF formula with standard peanut paste-based RUTF. They treated two groups of 6–23 and 24–59 month old children with SAM and the results revealed that the plant-based RUTF could be applied for the treatment of SAM in children older than 2 years. Although more clinical research is needed to optimize these RUTFs for targeting malnutrition issues among younger children, they have already showed promising signs of meaningful cost reduction in CMAM programs.

5 Herbs and Noncrop Plants

One major drawback in the widespread use of common crop plants as sources of efficient functional foods is that there are occasions when proper sources of a few biocompounds are not available. These compounds might include some essential amino acids, such as threonine, sulfur-containing amino acids, lysine, and tryptophan (Young and Pellett, 1994). It seems feasible to use different plant-based ingredients in a formulated mixture to provide an adequate amino acid balance to meet the minimum requirements of functional ready-to-use plants (Nabuumu et al., 2013); however, cultivation of a wide range of agricultural products in regions with the highest demand for RUTFs is a matter of concern. Therefore, identifying new natural sources for the development of more versatile and economical food formulations seems necessary.

A large body of literature and knowledge is available on the micronutrient composition of noncrops or wild vegetables, which generally reveals that these plants have a higher mineral and vitamin content than cultivated vegetables species. These species are traditionally known as medicinal plants because of their therapeutic and health benefits. However, their consumption in daily meals varies among regions and cultures of the world. Wild vegetables were consumed by the first European farmers and investigations into these species showed that almost all contained good levels of micronutrients; for example, *Verbena officinalis* was found to be an excellent source of calcium (3 mg/g) and magnesium (1.6 mg/g) (Guerrero

et al., 1998). This knowledge is not limited to Europe and researchers around the world have investigated various noncrop vegetative species that have been commonly used in local traditional meals. The importance of wild vegetables as sources of micronutrients was confirmed by Freyre et al. (2000) in South America, Sundriyal and Sundriyal (2001) and Gupta et al. (2005) in Asia, Vainio-Mattila (2000) in Tanzania, and Nesamvuni et al. (2001) in Africa. In Turkey, Turan et al. (2003) investigated 25 wild vegetables to evaluate their nutritional value and showed that these species contained similar concentrations of iron, manganese, zinc, and copper compared with cultivated species, such as spinach, lettuce, and cabbage. In addition, the authors also established that the nitrogen, potassium, calcium, magnesium, and protein content of the indigenous crops were meaningfully higher. In a separate study, the mineral concentrations of 32 noncrop Turkish plants were analyzed and the authors found that all species contained high levels of Al, Ba, Ca, Fe, K, Mg, P, and S (Özcan, 2004). Xu and Xu (2009) analyzed the bioactive elements in 22 Chinese therapeutic foods and herbs. The investigation was focused on a few rare trace nutritional and therapeutic elements including lanthanum in *Rhizoma Gastrodiae elatae* and *Fructus Crataegi*; strontium in *Radix Puerariae* and *Folium Ginkgo Biloba*; zinc (Zn) in *Flos Carthami Tinctorii* and *F. Crataegi*; and selenium (Se) in *Flos Lonicerae Japonicae* and *Portulaca Oleracea*. Their results showed that Chinese herbs (which were also considered as therapeutic foods) may be used as nutritional supplements for preventing and treating elemental deficiency.

In addition to their micronutrient richness, noncrop plants are also excellent sources of other nutritional and therapeutic compounds. Approximately 200,000 types of secondary biocompounds have been described in these plants, some examples of which include flavonoids, tannins, polyketides, carbohydrates, lignans, benzofuranoids, benzopyranoids, polycyclic aromatics, terpenoids, and alkaloids (Nguyen et al., 2013). The vitamin content of medicinal plant species in two districts of Vietnam was found to supply 21 and 14% of dietary (Ogle, 2001). In India, 36 out of 70 wild vegetables eaten by the tribal people were reported to contain high levels of vitamin A (Rajyalakshmi et al., 2001). Schönfeldt and Pretorius (2011) investigated the nutrition value of five wild leafy vegetables including raw misbredie (*Amaranthus tricolor*), pumpkin leaves (*Cucurbita maxima*), cat's whiskers (*Cleome gynandra*), cowpea leaves (*Vigna unguiculata*), and wild jute (*Corchorus olitorius*), which are traditionally consumed in rural areas of South Africa. The authors showed that in addition to microelements, such as magnesium, phosphorus, zinc, iron, and calcium, the studied species had acceptable (but varying) levels of vitamin B2 and β -carotene.

6 Cultivated and Wild Edible Mushrooms

Mushrooms have long been considered as medicinal herbs, although they are not exactly from the plant kingdom. There are about 3000 main edible fungi species, of which approximately 200 species are consumed by humans (Kalač, 2012). Approximately

100 of these species are cultivated by humans and a few of these are produced on an industrial scale (Chang, 1999). There is an increasing demand for mushrooms that are fresh, dried, extracted, and in food-integrated modes (Aida et al., 2009). This is in line with the soaring demand for raw materials of plant-origin and biocompounds, such as medicines and pharmaceutical precursors, food supplements, and cosmetics. Medicinal plants, and to a lesser extent medicinal mushrooms, are mainly collected from the wild or cultivated on small-scale farms. It has been estimated that 25% of the molecules used for pharmaceutical purposes in western countries are collected from medicinal plant species (Newman and Cragg, 2012). The two main reasons for the soaring mushroom demand is their relatively high nutritional value compared to most edible vegetables, and their economic and rapid large-scale cultivation (Petrovska, 2001). Whether cultivated by humans or collected from nature, mushrooms are known to contain valuable nutritional and therapeutic compounds. They have a very low dry matter content, typically ~100 g/kg. The chemical composition of mushrooms may vary depending on the species physiology or their cultivation conditions; however, they generally contain about 200–250 g of protein, 20–30 g of lipid and 80–120 g of ash per kg of dry matter. The energy level is also low among this species, typically 350–400 kcal/kg of fresh fruit bodies (Kalač, 2012). Mushrooms have a special place in traditional medicine and an especially long history in traditional Chinese medicine. Consequently, there has been a growing interest in studying the nutritional characteristics of mushrooms over the past few decades. Mushrooms are rich sources of protein, essential amino acids, and fiber, with low levels of fat and energy (Agrahar-Murugkar and Subbulakshmi, 2005; Barros et al., 2007; Mattila et al., 2002). Edible mushrooms contain significant levels of vitamins including B1, B2, B12, C, D, E, and many trace elements (Agrahar-Murugkar and Subbulakshmi, 2005; Heleno et al., 2010; Kalač, 2010; Mattila et al., 2002; Mdachi et al., 2004; Tuzen et al., 2007; Yilmaz et al., 2006). The mineral uptake mechanism in mushrooms is different to that of plants. Mushrooms are efficient at collecting chemical elements from their growing substrates and accumulating them in their tissues (Genççelep et al., 2009); for example, Kalač and Svoboda (2000) showed that mushrooms can absorb up to 90% of the iron present in their growing substrate. In another study, the mineral content of 30 wild-grown edible mushrooms were analyzed and the authors found that the macro- and trace elements were present at varying levels [e.g., Mg (0.90–4.54), Ca (0.17–8.80), K (12.6–29.1), Na (0.03–4.85), P (0.64–4.49), Zn (26.7–185), Fe (50.1–842), Cu (9.23–107), and Mn (5.54–135) in mg/g dry matter] (Genççelep et al., 2009). Many mushrooms are resistant to toxic minerals and are capable of accumulating these minerals in their tissues. Ouzouni et al. (2009) investigated the biochemical composition and mineral (Mn, Fe, Cr, Mg, Zn, Co, Ni, Cu, Cd, Pb, Al, Sn, and As) content of 10 wild edible fungi species. Their results showed that fungi could accumulate detectable levels of Mg, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb, and Cd and their detection method could not reveal the exact concentrations

of As, Al, and Sn, although it did not rule out their presence. The interesting point in their study was that the studied fungi were collected from forests and pasturelands in unpolluted areas and a distance from potential pollution sources. Therefore, caution should be taken when using wild mushrooms in food recipes, especially if they are collected from suspected polluted environments or near industrial zones.

Mushrooms are excellent sources of nutraceutical compounds, such as carotenoids, unsaturated fatty acids, tocopherols, ascorbic acid, and phenolic compounds (Barros et al., 2007, 2008; Elmastas et al., 2007; Ferreira et al., 2009; Pereira et al., 2012; Woldegiorgis et al., 2014). Reis et al. (2012) compared the chemical composition and nutritional values of the most consumed species of fresh cultivated mushrooms including *Agaricus bisporus*, *Pleurotus ostreatus*, *Pleurotus eryngii*, *L. edodes*, and *Flammulina velutipes*. All of the species showed varying concentrations of macronutrients, proteins, sugars, tocopherols, monounsaturated fatty acids, and polyunsaturated fatty acids. They also showed variations in the saturated fatty acid content and total energy values. The authors also found that the chemical composition varied between cultivated and wild mushrooms of the same species, which could be due to their different growing conditions. The synergistic and cumulative effect of these biocompounds is responsible for many of the therapeutic and health benefits of mushrooms, such as their antioxidant capacity (Cheung et al., 2003; Elmastas et al., 2007; Woldegiorgis et al., 2014), their antitumor activity (Ajith and Janardhanan, 2007; Zaidman et al., 2005), their ability to prevent hypertension and hypercholesterolemia (Jeong et al., 2010), and their antimicrobial and antifungal properties (Hearst et al., 2009; Oyetayo, 2009; Ramesh and Pattar, 2010). Barros et al. (2008) analyzed the nutritional and therapeutic value of 11 cultivated and wild mushroom species from Portugal. The analysis encompassed proteins, fats, ash, carbohydrates, fatty acids, tocopherols, phenolics, flavonoids, carotenoids, and ascorbic acid contents. The antimicrobial properties of the mushrooms were also investigated against fungi, Gram-positive, and Gram-negative bacteria and their results revealed that there were some differences in the types and concentrations of biocompounds between the wild and cultivated species; however, the authors found that the antimicrobial properties were similar. They suggested that more research on mushrooms could lead to the generation of new therapeutic foods. Kalač (2009) reviewed the chemical composition and nutritional value of wild fungi species, as well as their capacity to accumulate detrimental trace elements. They also assessed the presence of detrimental constituents in edible mushrooms. More recently, Kalač (2012) thoroughly reviewed the current literature on various nutritional and biochemical aspects of edible mushrooms.

The high protein and amino acid content in mushrooms is the reason why they are classified as protein foods. Petrovska (2001) analyzed the protein distribution of 52 edible mushrooms and found that protein content in the studied samples were relatively

high (14–52.1%, dry basis) with the main protein fractions including albumins (24.78%), globulins (11.46%), glutelins (11.46%), glutelin-like material (7.43%), prolamins (5.67%), and prolamin-like material (5.31%). When considering plants for the supply of nutrients and biocompounds it should be noted that plants cannot synthesize four essential amino acids lysine, tryptophan, methionine, and phenylalanine. This is one of the limitations of completely substituting animal-based ingredients with plant-based ones. However, as the fungi kingdom is evolutionary closer to animals, almost all of the known animal amino acids can be found in edible mushrooms. A few studies have specifically investigated this issue and analyzed the amino acid content of edible cultivated or wild mushrooms. Kim et al. (2009) analyzed the free amino acid composition of 10 popular edible and medicinal mushroom species in Korea including *P. ostreatus*, *Arabidopsis bisporus*, *F. velutipes*, *P. eryngii*, and *L. edodes*. The average total free amino acids were reported to be 91.13 mg/g of dry matter and all essential amino acids (with the exception of tryptophan) were present in the amino pool of the studied mushrooms.

Mushrooms or macrofungi are among the most powerful decaying organisms in nature, and together with bacteria they perform most of the recycling in the wild. Their kingdom is evolutionarily close to animals, which leads them to share many physiological mechanisms with animal cells. They are fast and easy to grow, especially under controlled environments and on large scales. Hence new opportunities may be introduced to harness these capacities. This may be particularly noteworthy in low-income, agricultural communities as it could help to turn lignocellulosic or value-less agricultural wastes into products with enhanced nutritional parameters and added economic value. Finally, it should be noted that the application and acceptability of medicinal plants and mushrooms in medicine and food is largely influenced by culture, philosophy, personal preferences, and the diversity of flora in each region (Magoro et al., 2010).

7 Genetic Modifications to Enhance the Nutritional Quality of Plants

The nature's capacity to address the unprecedented human demand for food, feed, and energy is rapidly declining. In addition to the amount of food required, there is also a demand for more nutrition in the daily diet, particularly through the inclusion of plant types in recipes. The demand is even higher than that of cereals in many developing countries. Therefore, possible solutions should be evaluated to address the ever-growing demand for more added values in foods. One possible solution might be to increase the production and productivity of nonstaple foods through agricultural research; however, this may not be feasible because the number of, and variation in these, plants are overwhelming. A better option might be to enrich plant-based staple foods (i.e., cereals)

with more nutritional value (Bouis, 2007). This strategy is already being pursued around the world by scientific, economic, global, and regional decision making bodies. Therefore, in addition to finding renewable substitutes for the already-depleting natural resources, it is envisioned that strategies will be devised to improve the productivity and adaptability of natural living resources, (e.g., plants and animals). Nevertheless, when planning and developing novel characteristics or improving the existing qualitative and quantitative traits within plants or animals (i.e., increasing the concentrations of biocompounds to improve the nutritional quality of crop plants), there will be some limitations and proper planning and tools should be available. The genetic manipulation of organisms through conventional breeding programs or modern biotechnological techniques appears to be a feasible solution. Conventional breeding methods have been formally practiced for almost half a century; however, this approach is basically dependent on existing genetic resources, and the absent or limited access to rich genetic resources for higher levels of nutritional elements and biocompounds in the plant kingdom would make it difficult to make meaningful progress in the short term. The interwoven nature of the genetics of metabolic pathways with plant phenotype and performance, and the lack of site-specific expression of these traits complicates the task even further. Conversely, modern genetic engineering approaches permit site-specific genetic manipulations in the plant genome, which can introduce a particular foreign gene or improve the expression profile of a naturally occurring gene within tissues of interest (i.e., seeds or fruits). In addition, genetically engineered traits could then be easily transformed into other plant genotypes and programmed to function synergistically with important agricultural traits. Therefore, modern genetic engineering has enabled the introduction, isolation, and improvement of nutritional properties in plant- and animal-based foods, which is encouraged by market demand (from consumers and business entrepreneurs) and scientific advances (Charpentier and Doudna, 2013; Holland and Johnson, 2012; Tucker, 2003; Twine, 2010). The genetic engineering of animals and microorganisms has been more medically-oriented and aimed at studying disease, developing new drugs, or testing newly-developed medicines. Conventional and biotechnological approaches in plants have historically focused on: (1) making plants more resistant to biotic and abiotic stresses (Al-Khayri et al., 2016; Ammann, 2005; Bhatnagar-Mathur et al., 2008; Bowne et al., 2011; Collinge, 2016; Dita et al., 2006; Lombardo et al., 2016; Roy et al., 2014); (2) improving crop yields by developing higher-yielding cultivars and decreasing agricultural wastes by promoting postharvest traits (Aghdam et al., 2015; Edgerton, 2009; Huang et al., 2004; Qaim and Zilberman, 2003); (3) improving the nutritional value of plants by increasing the concentration of naturally existing compounds or elements (Alós et al., 2016; Botella-Pavía and Rodríguez-Concepción, 2006; Hefferon, 2015; Tien Lea et al., 2016; Paine et al., 2005); (4) introducing de novo biocapabilities for the production of novel pharmaceuticals (Chandler and Lu, 2005; Sack et al., 2015); and (5) enhancing other traits

(largely in ornamental species) to promote their marketability and price (i.e., introducing propagation, new coloring themes, and longer postharvest freshness) (Nishihara and Nakatsuka, 2011; Tanaka et al., 2009). Limited information exists on the genetic modification of edible macrofungi and mushrooms at this time. The concept of mushroom biotechnology is now more concerned with fermentation, mushroom bioprocesses, and biology (Cheung, 2008). One of the reasons for this might be related to the complicated nature of fungi reproduction as they produce millions of spores to transfer their genetic content, which creates biosafety concerns. Therefore, although there are many advances in transgenic technology that aim to safeguard GM procedures, there are still real concerns about the transfer and distribution of modified genetic material to unknown or unwanted fungi. The genetic engineering of plants could be studied under two major groups: crop plants and noncrop plants. Any plant species cultivated by humans to satisfy food or industry requirements or to provide raw materials or livestock feed is considered to be a crop plant. Noncrop plants refer to any species that are not commercially cultivated by humans or are largely collected from nature (including wild plants and most medicinal plant species). The qualitative improvement of livestock feed (or so-called forage crops) has a positive effect on livestock nutrition and productivity characteristics (Cherney and Cherney, 2002). This approach could indirectly and efficiently alleviate malnutrition in many underdeveloped regions because animal-based foods are typically more nutritious in terms of microelements, such as iron, vitamins, and protein content when compared with plant-based meals (Randolph et al., 2007). However, the evaluation of the indirect effects of livestock feed enrichment on human health is a multidisciplinary discussion and is not within the scope of this current chapter. The focus here is on the genetic manipulations aimed at enhancing the nutritional quality in crop plants, which are directly consumed by humans with minor processes.

The genetic modification programs aimed at improving the nutritional value of human food crops are mainly targeted at: (1) increasing the abilities of plants to accumulate higher levels of micronutrients (Brinch-Pedersen et al., 2007; Grusak, 2002; White and Broadley, 2005); (2) promoting the bioavailability of plant-based nutrients by either reducing antinutritional biocompounds in the storage tissues of crop plants (Bhullar and Gruissem, 2013; Bock et al., 2009; Clarke and Wiseman, 2000) or increasing the levels of biocompounds that enhance nutrient assimilation in humans; (3) increasing the levels of naturally-occurring amino acids or introducing missing essential amino acids in plants (Galili and Höfgen, 2002; Galili et al., 2016; Leuchtenberger et al., 2005); and (4) de novo production of pharmaceutical biocompounds, which help promote human health and act as natural therapeutic factors (Fahad et al., 2015; Floss et al., 2007; Tekoah et al., 2015). The results of such approaches are known as genetically modified foods (GMFs). Table 3.3 summarizes a few examples of genetic manipulations in plants to improve their nutrient quality.

Table 3.3: Examples of genetic modification in crop plants for higher nutrient quality.

Plant	Tissue/Product	Modification Type	Target Compound	References
Canola	Seed/oil	NAP	Carotenoids	Ravanello et al. (2003); Shewmaker et al. (1999)
Bean	Grain	AA	Lysine	Falco et al. (1995)
		NAP	Folate	Ramírez Rivera et al. (2016)
		ANS	Low phytate	Panzeri et al. (2011)
		AA	Methionine	Hanafy et al. (2013)
Potato	Tuber	NAP	Zeaxanthin carotenoid	Lopez et al. (2008); Römer et al. (2002)
		AA	Protein (albumin)	Chakraborty et al. (2000)
			Methionine	Di et al. (2003); Zeh et al. (2001)
Rice	Grain	NAP	Carotene	Gayen et al. (2015); Ye et al. (2000)
			Ferritin	Vasconcelos et al. (2003)
			Provitamin A	Paine et al. (2005)
			Lactoferrin	Nandi et al. (2002); Rachmawati et al. (2014)
		ANS	Low phytate	Ali et al. (2013); Kuwano et al. (2009)
			High phytase	Hamada et al. (2006); Hong et al. (2004)
		AA	Methionine	Nguyen et al. (2012)
			Lysine	Kawakatsu et al. (2010)
Wheat	Grain	NAP	Carotenoids	Cong et al. (2009)
		ANS	High phytase	Brinch-Pedersen et al. (2006)
Maize	Kernels	NAP	Provitamin A	Aluru et al. (2008)
			Carotenoids	Wurtzel et al. (2012)
			Vitamin C (ascorbic acid)	Chen et al. (2003)
		ANS	High phytase	Chen et al. (2008); Gao et al. (2012)
			Low phytate	Pilu et al. (2003); Shukla et al. (2004)
		AA	Lysine	Azevedo and Arruda (2010); Huang et al. (2005); Tang et al. (2013)
			Storage protein (amarantin)	Rascón-Cruz et al. (2004)
Soybean	Seed/oil	NAP	Fats and oils	Clemente and Cahoon (2009); Kinney and Clemente (2005); Lee et al. (2007)
			Vitamin E	Sattler et al. (2004); Van Eenennaam et al. (2003)
			High phytase	Bilyeu et al. (2008); Chiera et al. (2004); Li et al. (2009)
		ANS	Low phytate	Yuan et al. (2007)
		AA	Methionine	Hanafy et al. (2013)
			Tryptophan	Kita et al. (2010)
			Lysine	Falco et al. (1995)

(Continued)

Table 3.3: Examples of genetic modification in crop plants for higher nutrient quality. (*cont.*)

Plant	Tissue/Product	Modification Type	Target Compound	References
Tomato	Fruit	NAP	Fatty acid composition Folate Carotenoids Provitamin A Zanthophylls Flavonoids	Cook et al. (2002) de la Garza et al. (2004) ; Hossain et al. (2004) Diretto et al. (2007) ; Fraser et al. (2002, 2009) ; Wurbs et al. (2007) Römer et al. (2000) Dharmapuri et al. (2002) Bovy et al. (2002)
Cauliflower	Leaf	NAP	β-Carotene	Lu et al. (2006)

AA, Amino acids; ANF, antinutrient factor; NAP, nutrients absorption promoter.

8 Micronutrients

If conventional and biotechnological plant-breeding methods are fully exploited by public health communities, they could be promising approaches to address micronutrient deficiencies in the human population ([Graham et al., 2001](#)). The biofortification of staple food crops is a term that refers to increasing the bioavailable nutrient content in crop plants using different plant breeding methods. The biofortification is a sustainable and efficient solution to address the nutrition problem in underdeveloped communities. Introducing improved crop cultivars empowers these communities to produce their own enriched foods in the long term ([Welch and Graham, 2004](#)), and the development of micronutrient-enriched crops would contribute to the productivity of such plants when their seeds are sown in micronutrient-poor soils (which are typically found in underdeveloped regions of the world including many African counties). Therefore, this approach contributes to the correction of malnutrition, significantly promotes agricultural activities, and leads to local economic growth in these areas ([Graham et al., 2001](#)).

Knowledge of the physiological and molecular mechanisms of element uptake, transport, and accumulation in plants is growing rapidly, which is pivotal for successful plant breeding and micronutrient alleviation programs. For example, the concentration of elements in plant tissues differs among different species regardless of their natural growing environment ([Broadley et al., 2004](#)), which might be the result of constraints imposed by evolutionary processes. A few differences in crop plants have been linked to major nutrient deficiencies in the human population. For example, calcium, magnesium, iron, and zinc are found in higher concentrations in dicotyledons than monocotyledons ([Broadley et al., 2003](#); [Kabata-Pendias, 2010](#)) and therefore populations who replaced their vegetable-, fruit-, and legume-rich diets with monocot-rich foods (i.e., cereals) showed more acute micronutrient deficiencies ([Graham et al., 2001](#)). Interestingly the mineral distribution within different

tissues of the same plant also varies and is effectively governed by genetic mechanisms. For example, the concentrations of P, N, Se, S, Cu, and Cr are higher in below-ground tissues or plant roots while Ca, Si, Mg, Zn, Ni, and K are more abundant in above-ground tissues (Hodson et al., 2005; Karley and White, 2009). Therefore, improving the total nutrient content in crop plants could be one step toward increasing their bioavailability in human foods. Nevertheless, the conventional or biotechnological improvement of element content in most cultivated crop plants is still not a straightforward task. In addition to the complications associated with biotechnological methods, there are several physiological obstacles to be overcome when attempting to increase the concentration of metal elements in plant tissues. These so-called barriers are in fact naturally-occurring tightly-controlled homeostatic mechanisms that regulate element absorption by plant roots, translocation and redistribution, and the accumulation of elements within edible plant tissues (Welch and Graham, 2004). Karley and White (2009) reviewed the literature regarding the identification and characterization of molecular mechanisms that are responsible for taking up and redistributing metal elements between plant organs, cell types, and subcellular compartments. They highlighted the limitations of element distribution within the plant that led to lower concentrations of much-needed nutrients in edible parts of the plant.

9 Antinutritional and Promoter Factors

Plant foods contain substances that affect the bioavailability of micronutrients to humans. These compounds are known as ANFs or nutrient inhibitors, and by definition they are substances that contribute to the low bioavailability of essential plant elements through impairment of absorption in the human gut (Raes et al., 2014; White and Broadley, 2005). The existence and concentration of these substances is considerably varied among plant species and between edible portions of a plant (especially grains), and is influenced by both genetic and environmental factors (Erdal et al., 2002; Guzmán-Maldonado et al., 2000; Kumar et al., 2010; Vreugdenhil et al., 2005; White and Broadley, 2005). Important ANFs include phytate in whole legume seeds and cereal grains; different types of fibers, such as hemicellulose, cellulose, and lignin in whole grain cereals; tannins and phenolics in coffee, tea, sorghum, and beans; oxalic acid in spinach leaves; hemagglutinins, such as lectin in wheat and legumes; and oxalate (Graham et al., 2001; Mendoza, 2002; White and Broadley, 2005). However, the ANFs are not necessarily of natural plant metabolites, rather environmental pollutants, such as cadmium, mercury, and lead, which are absorbed by the plant and accumulated in the roots and leaves, might act as nutritional inhibitors in the human body. For instance, phytic acid may bind to positively charged proteins, amino acids, and minerals to make complexes that are insoluble and difficult to hydrolyze during digestion (Suhairin et al., 2010). Therefore, modifying the concentrations of known ANFs in plant-based foods to improve their bioaccessibility is a sustainable strategy when combined with enhancement of the plant's mineral content (Hotz and Gibson, 2007). However, there

are biological limitations and food safety concerns when manipulating the levels of these compounds using conventional or biotechnological methods. In other words, many of these ANFs are in fact major plant metabolites that play important roles in plant physiology and metabolism. For example, phytin is the main storage form of phosphorus in mature seeds and is an influencing compound that is required for early seed germination and seedling growth. It also determines the level of mineral reserves and affects the seedling vigor and viability (Kim et al., 2002; Konishi et al., 2004). Some ANFs (i.e., polyphenols and plant fibers) have roles in human food safety as they act as anticarcinogens, dietary antioxidants, or by reducing some diseases (i.e., diabetes) (Kaczmarczyk et al., 2012; Kendall et al., 2010; Pandey and Rizvi, 2009; Vauzour et al., 2010; Visioli et al., 2011). They also help to prevent or reduce the absorption of toxic compounds or elements, such as heavy metals (Clemens, 2006). From an agricultural perspective, developing plant seeds with lowered ANFs might negatively affect agricultural productivity in regions where the soil is not fertile enough to compensate for the lowered concentration of compounds that are essential for plant germination and development. Hence innovative solutions should be applied with caution when modifying the ANF content in major crops.

The importance of managing ANFs in plant-based foods has urged the scientific community not to wait for advancements in complicated biotechnological methods. The conventional plant breeding techniques have been used to develop plant mutants that accumulate low levels of known ANFs (i.e., several low phytic acid mutants). This approach has mainly involved the selection of plant genotypes with low ANF contents and crossing them with cultivars or lines with higher mineral contents (Graham et al., 1999). The resulting hybrids or lines then undergo further selection steps to obtain more stable hybrids. Raboy (2002) reported the isolation of cereals and legumes with low-phytic acid mutations, which were subsequently used to breed first-generation low-phytate hybrids of maize, barley, rice, and soybean. Phytic acid contents were reduced by 50%–95% in mutant compared with nonmutant seeds. Since the ANF content of a plant influences the availability of targeted minerals (i.e., iron, zinc, and magnesium), the breeding programs try to simultaneously improve the mineral content while reducing the corresponding ANFs. Thus modified plants with *lpa* mutations often show higher or similar concentrations of seed Fe, Zn, and Mg to wildtype (Graham et al., 1999; Guttieri et al., 2004; Lott et al., 2004; Raboy et al., 2001; Wilcox et al., 2000).

Transgenic approaches have also followed the same trend for improving the bioavailability of essential trace elements (i.e., iron and zinc) in plant-based foods. Thus advanced molecular genetics and biotechnological techniques are applied to reduce the concentrations of ANFs and increase the concentrations of promoter substances and the uptake of plant minerals (Lucca et al., 2006; White and Broadley, 2009). Phytate is the ANF that has received the most attention due to its meaningful inhibitory effects on iron and zinc absorption. Three main approaches have been implemented to reduce phytate levels in plants (Ali et al., 2013; Raboy, 2007): (1) impairing the expression of genes that encode enzymes involved in the

synthesis or sequestration of phytate. This has been applied for the down regulation of phytate synthesis in rice, soybean, and maize and led to a decrease in phytate seed levels (Greiner et al., 2007; Kuwano et al., 2009; Panzeri et al., 2011; Pilu et al., 2003; Shukla et al., 2004); (2) reducing the accumulation or storage of phytates through the overexpression of enzymes that catalyze the hydrolysis of phytic acid or phytases within the edible parts of crop plants including wheat (Brinch-Pedersen et al., 2006), rice (Hamada et al., 2006; Hong et al., 2004), soybean (Bilyeu et al., 2008; Chiera et al., 2004; Drakakaki et al., 2005; Li et al., 2009; Yuan et al., 2007), and maize (Chen et al., 2008; Gao et al., 2012); and (3) introducing heat-stable phytase enzymes that can resist postharvest and cooking processes (i.e., milling or boiling) that may further improve nutrient absorption even after consumption (Brinch-Pedersen et al., 2006; Holm et al., 2002; Mendoza, 2002).

Facilitating nutrient absorption by enriching plant-based foods with biocompounds has also shown promising results. Nutrient absorption promoters (NAPs) are typically organic in nature and tend to improve plant food quality by facilitating the absorption of essential elements in the human intestines (White and Broadley, 2005). The occurrence and concentration of NAPs are also influenced by genetic and environmental factors and may vary in different parts of the same plant (Frossard et al., 2000; Welch and Graham, 2004). The most abundant compounds in the plant kingdom are plant metabolites that play specific roles during plant growth, development, environmental response, and defense. The substances that promote the assimilation of iron, zinc, iodine, and vitamins (which are more abundant in plants) may include organic acids (e.g., fumarate, malate, citrate, and ascorbic acid), fats and lipids, β -carotene, amino acids (i.e., cysteine), metal-binding proteins (e.g., ferritin and lacto-ferritin), and selenium. Other NAPs, such as hemoglobin and long-chain fatty acids (e.g., palmitate) are mainly found in animal dietary sources and also contribute to mineral absorption. A group of nondigestible food ingredients known as prebiotics, may also enhance the growth of some beneficial microorganisms (probiotics) in the human gut, which leads to enhanced food digestion and an increased chance of nutrient absorption. Various genetic modification tools have been used to produce higher concentrations of promoters in many crop plants (Table 3.3). As for antinutrients, the main strategy is to enhance the synthesis of a targeted biocompound through upregulation of the genes responsible for its biosynthesis. Another method involves the down regulation of genetic pathways that degrade beneficial compounds within plants. Manipulating the intercellular transportation of such compounds to prevent their accumulation in nonedible tissues before harvest has been also considered.

10 Essential Amino Acids

Amino acids are the building blocks of proteins and they occur naturally in free and protein-bound forms in animals, plants, fungi, and eukaryotic microorganisms. Amino acids are composed of a carboxylic acid group ($-\text{COOH}$) and an amino group ($-\text{NH}_2$) (Barrett, 2012)

and they are among the most important biomolecules in animals and plants. Humans requires approximately 20 amino acids to maintain growth and metabolism. When the concentration of one or more of these amino acids is not maintained at the minimum required level, the human body is urged to catabolize other amino acids and use them as energy sources or building blocks to synthesize essential proteins. This mechanism, hinders proper and sufficient protein synthesis in the long term and eventually leads to protein–energy malnutrition syndrome. The WHO estimated that ~30% of people in the developing world suffer from this syndrome, which can seriously affect mental and physical health in children (WHO, 2007). Humans can manufacture most amino acids using their own carbon, nitrogen, and energy sources; these include asparagine, alanine, serine, cysteine, tyrosine, glutamine, proline, glycine, taurine, glutamic acid, aspartic acid, hydroxyproline, ornithine, carnitine, c-aminobutyric acid, citrulline, glutathione, and cysteine. However, due to metabolic limitations, humans are unable to synthesize nine amino acids *de novo* and these amino acids must be obtained from the daily diet. These are grouped as essential amino acids and are methionine, histidine, lysine, valine, phenylalanine, tryptophan, isoleucine, threonine, and leucine (Wu, 2009). Although animal-based diets can provide most essential amino acids, plants still form a considerable portion of the human diet and in many underdeveloped countries plants are the first and major staple food. Plants synthesize various amino acids for growth and development; however, the quantities of a few essential amino acids (i.e., threonine, lysine, tryptophan, and methionine) are very low in plant-based foods, especially cereals and legumes. Therefore, enriching plant-based foods with certain amino acids has long been a target in crop plants.

Conventional breeding and genetic engineering have been used to improve the amino acid content of crop plants. As mentioned earlier, the limitations in the rich genetic resources and the complicated nature of many agronomic and physiologic traits in plant, means that conventional breeding cannot meaningfully contribution to this aim in the short term. For instance, when the concentration of a selected amino acid has been increased through conventional breeding methods, it has also led to a significant reduction in crop yield (Imsande, 2001; Streit et al., 2001). The factors that might hinder the efforts include the regulatory mechanism of feedback inhibition, which establishes a balance between the amount of amino acids available and the activities of their respected synthetic pathways (Hong et al., 2000). Consequently, the natural concentration of an amino acid is tightly regulated by this mechanism and any attempt to manipulate the balance could lead to negative feedback on the plant's performance and phenotype. Another obstacle is related to the predefined mission of amino acids in the cellular metabolism, which further limits their constant level in the cell. For example, one of the roles of amino acids is to provide precursors for biocompounds that affect the plant growth, development, environmental stress response, and cellular energy production under energy starvation periods by degradation in the Krebs cycle (TCA) (Baena-González et al., 2007; Hildebrandt et al., 2015; Pratelli and Pilot, 2014;

Zeier, 2013). Amino acids are also known to be precursors for plant vitamins (B1, B2, B3, B5, B7, B9, and E) and they play different roles in biosynthesis and metabolism by becoming incorporated into vitamin backbones or as sulfur, amino, or carbon group donors. This is important in terms of plant physiology and human diet (Miret and Munné-Bosch, 2014). There is a need for more advanced techniques to delicately manipulate the interconnected physiologic processes and to this end, biotechnological techniques may offer promising solutions.

Two genetic engineering approaches have been adopted to increase the levels of essential amino acids in the edible tissues of crop plants; a combination of these techniques might produce better results under certain circumstances. The first method is to improve the content of the amino acid of interest by overexpressing its biosynthesis pathway. As mentioned earlier, amino acid metabolism is typically regulated by a feedback signaling system (the feedback inhibition cycle), which sets a balance between the amino acid content and its production rate. Thus targeting and upregulating a feedback-insensitive route is favorable for the expression of amino acids. The second approach is to reduce the catabolism of amino acids by suppressing or downregulating their catabolic enzymes. This method has been successfully used to increase lysine and methionine, and to lesser extend tryptophan and threonine (Zeh et al., 2001). In addition to increasing a singular amino acid, the possibilities of improving storage proteins, which are rich in one or more amino acids of interest, has also been investigated. This approach is the method of choice when engineering grain crops (Amir, 2010; Amir et al., 2012). Contrary to the latter technique, it would be possible to decrease a seed protein that is not rich in the amino acids of interest and steer the biosynthetic machinery toward storage proteins with a higher nutritional value (Azevedo and Arruda, 2010). Table 3.3 provides examples of crop plants that have been genetically manipulated to improve the protein and amino acids content in their edible tissues.

11 Ethical Issues

Genetically modified organisms (GMOs) have been widely criticized by the public, environmental activists, and health authorities due to concerns over introducing unknown genetic material into target genomes (Bawa and Anilakumar, 2013; Frewer et al., 2013; Lunshof, 2015). However, the advantages of these technologies cannot be ignored, and the focus of many research projects is now on improving the accuracy of genome manipulations while decreasing the food and feed safety concerns. New biotechnology techniques have been developed that simply edit the genome and do not require invasive and extensive manipulations of the target genome. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) technique (Cong et al., 2013) and transcription activator-like effector nucleases (TALENs) (Sun and Zhao, 2013) are the two prominent examples of such techniques and they are supposed to perform precise and safe editing in various

eukaryotic genomes (Gaj et al., 2013). These technologies have already revolutionized the way researchers think about enhancing food. It appears that new plant cultivars, which are developed through these technologies, will be subjected to the same regulations and concerns as the current transgenic crops are. Therefore, genome editing will hopefully help the plant geneticists and biotechnologists to avoid the public concerns about GMOs (Puchta and Fauser, 2013). Although genome editing technology is still in its infancy, it has already been applied in crop plants, including rice (Zhang et al., 2014), barley (Gurushidze et al., 2014; Lawrenson et al., 2015), tomatoes (Brooks et al., 2014), and maize (Liang et al., 2014). However, patience should be practiced by all stakeholders including the public, policy makers, and scientific communities so that more insight can be gained into the true nature and consequences of genetically manipulating staple foods. The emergence of novel and environmental friendly techniques could also contribute to the general acceptance of GMOs in the near future.

12 In Silico Tools for RUTF Development

The high cost of commercial RUTF products and the growing demands for therapeutic foods have encouraged the use of locally produced and acceptable ingredients and increased their production in developing countries (Lagrone et al., 2010). However, there is a need to use innovative tools when developing new RUTF formulas to meet the cost, nutritional value, availability, stability, and consumer preferences. The use of programming and computational tools would save considerable time and costs and lead to higher levels of acceptability among authorities and communities. Dibari et al. (2012) developed a method based on linear programming to formulate a RUTF prototype in East African. The program was set to predict a formulation with the lowest price and weights using regional plant supplies including soybean, maize, sorghum, plant oil, and sugar, while fulfilling the FAOs codex guidelines for RUTF nutrition and energy requirements. Their method was found to have good accuracy (up to 90%) when compared to the laboratory results of the designed RUTF. Weber and Callaghan (2016) had tried to optimize the protein quality, cost, and acceptability of RUTFs through a linear programming approach. The prototypes of their predicted formula and a standard RUTF were passed for informal acceptability testing in the Manary Food and Nutrition Lab, and then sent to Ethiopia, Ghana, and Pakistan for formal acceptability testing. Their results were positive and reassuring in terms of their acceptability and consumption ratios.

13 Food Processing Techniques

The processing techniques used for the production of RUTFs should be considered as important as the ingredient selection. Using various processing techniques improves the nutritional value and makes the nutrients and energy in the food more readily available to humans, and this is especially true when using raw plant ingredients. Ingredient-processing

methods are extremely diverse and each may serve a specific function during the production of a plant-based food product. For example, plants, such as rice and sorghum contain enzymes or digestion-inhibiting biocompounds (i.e., protease, inhibitor phytate, or poisonous phytotoxins in cassava) that negatively affect the nutritional efficacy of the final food or might cause health risks. Therefore, plants should be properly processed to be safe and efficient to use in foods. Processing methods include roasting, dehulling, boiling, soaking, pounding, fermenting, and enzymatic hydrolysis (Hotz and Gibson, 2007). It is recommended that a combination of processing techniques be applied to gain the best results; however, caution should be taken when applying cooking methods to prevent a loss of quality or chemical composition change, especially for homemade foods. Reports have suggested that there is an increase in moisture content during the home cooking of traditional foods, while minerals, fats, proteins, and ash decrease during the cooking process (Schönfeldt and Pretorius, 2011). Conversely, the loss in nutritional value of a crop plant might not be related to either the plant genetics or the food cooking techniques, but rather to mediatory processes, such as harvesting or postharvesting methods. For example, most cereals are typically milled or polished before being used in foods; however, the concentrations of essential micronutrients (i.e., Fe, Zn, Cu, S, and Se) are higher in the embryo and bran (Raes et al., 2014; Vasconcelos et al., 2003). Milling and polishing processes remove large portions of these elements from the diet (Gehring et al., 2013; Serna-Saldivar, 2016).

14 Conclusions and Future Perspectives

Genetically modified crops for higher yield or enhanced resistance against abiotic and biotic stresses are now cultivated on over 300 million acres in more than 25 countries. Despite there being concerns over GM foods in some communities, the acceptance of these products is growing. This is especially true in developing countries that have the highest rates of population increase and demand for food. However, breeding staple foods, which are enriched with a higher content of minerals and vitamins to economically and sustainably alleviate the problem of hidden hunger or undernutrition, has not yet received enough attention. Thus, in order to properly address the problem, effective interaction among all stakeholders including policy makers, public NGOs, international regulatory and observer organizations, and various scientific disciplines including biologists, nutritionists, and biotechnologists seems inevitable.

The application of many conventional and modern genetic engineering approaches have made considerable progress toward alleviating hunger in many parts of the world; however, more efforts are needed to expand the scope of such strategies to elevate the nutritional value in most common crop plants, such as wheat, maize, or rice. In addition, the new crop varieties with enhanced nutritional qualities should be clinically evaluated. Target societies must be educated prior to crop release to help farmers and consumers understand the advantages of the new fortified crops, and to what extent they can contribute to their community's health

and development. The scope of research and development programs should also be extended to cover the “orphan crops,” such as millet and sorghum, which are commonly grown as staple foods in many underdeveloped communities.

Finally, a few challenges in the biofortification of many common crops should be addressed, including how to reach the target micronutrient levels while offering high yields, disease resistant, and acceptable marketability. Moreover, rapid and low-cost screening methods should be developed to ensure the quality and safety of crops. Interaction between genetics and the environment in each geographical region is also a matter of concern that leads to higher costs and longer development times in region-specific plant varieties. In spite of all the current limitations, genetically engineered plant foods are still among the most promising solutions to eliminate hunger and undernutrition in the future.

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Approved Genetically Engineered Foods: Types, Properties, and Economic Concerns

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1 Introduction

Food is one of the essential factors for sustaining life as it provides the basic energy requirement for living. In the past few decades, exponential population growth, environmental degradation, and climate change have created significant challenges, such as reduced availability of agricultural land and increased pollution rates associated with food production. For this reason genetically modified foods (GMFs) are seen as sustainable alternatives to enhance food availability and quality (Gurău and Ranchhod, 2016).

GMFs refer to foods produced from genetically modified organisms (GMOs) (i.e., microorganisms, plants, or animals) using various biological techniques. The term GMOs was defined by the World Health Organization (WHO) as “organisms (i.e., plants, animals, or microorganisms), in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination.” Similarly, the Food and Agriculture Organization (FAO) of the United Nations defined GMOs as products which do “not occur naturally by mating and/or natural recombination” (Hudson, 2017; Zhang et al., 2016a). The first GM crop was approved in 1994 and since then the number of approved GM crops has increased and been relatively constant over the course of the past two decades (Fig. 4.1). At present over 357 GM traits have been approved in various crops with the most frequent being identified in canola, maize, cotton, soybean, and potato.

In 2015 the Food and Drug Administration approved the first genetically modified (GM) animal for human consumption: a new milestone for GMFs (Wong and Chan, 2016).

GM crops are present in the human food chain either directly as food or indirectly as animal feed. The biological methods used for the genetic modification are referred to as

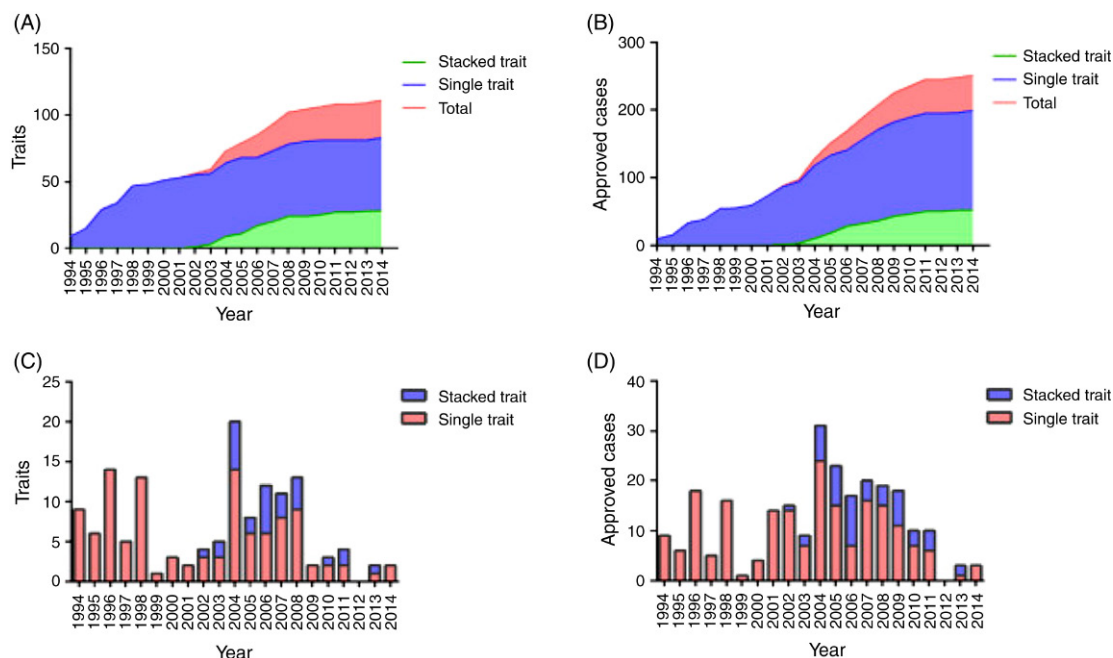


Figure 4.1: Annually Introduced Genetically Modified (GM) Crop Traits and Approved Cases for Food/Feed Use.

(A) Accumulated GM crop traits. (B) Accumulated GM crop approved cases. (C) New GM crop traits introduced each year. (D) New approved cases introduced each year ([Lin and Pan, 2016](#)).

recombinant DNA technology. These technologies were initially designed to manipulate bacterial DNA, but have since been adjusted to allow the incorporation of genes encoding specific traits to permit the production of various phenotypes. For example, GM crops can be produced to demonstrate herbicide resistance, insect resistance, and pathogenic virus resistance. The future applications of GM crops may lie outside the food sector, such as in the biomedical industry (i.e., vaccines and monoclonal antibodies) or in industrial products (i.e., packaging) ([Moglia and Portis, 2016](#)). The global vaccine market is diversified and faces constant challenges. Genetic modification techniques could offer a solution to this problem and facilitate the design and development of novel vaccines. [Ramezanpour et al. \(2016\)](#) reported the significant number of cooperative patent classification codes for GM vector-based vaccine patents ([Fig. 4.2](#)). In clinical trials, 26% of GM vaccine trials use vector-based candidate vaccines, mostly in cancer, malaria, HIV, and tuberculosis.

The FAO of the United Nations reported that the global demand for food would increase by 40% in 2030; therefore, the need for GM crops will also increase. [Moglia and Portis \(2016\)](#) reported that the main targets for marketing a GMF. These targets refer to the advantages

Table 4.1: Summary lists of the genetic modified foods (GMFs) (Zhang et al., 2016a).

Scientific Names	GM Traits	Trade Names
Apple (<i>Malus domestica</i>)	Antibiotic resistance and a nonbrowning phenotype	Arctic “Golden Delicious” apple and Arctic “Granny Smith” apple
Eggplant (<i>Solanum melongena</i>)	Lepidopteran insect resistance and antibiotic resistance	BARI <i>Bt</i> Begun-1, -2, -3, and -4
Argentine canola (<i>Brassica napus</i>)	Antibiotic resistance, modified oil/fatty acids, male sterility, glufosinate herbicide tolerance, fertility restoration, oxynil herbicide tolerance, and glyphosate herbicide tolerance	Laurical canola, Optimum GLY canola, Roundup Ready canola, LibertyLink Independence, InVigor canola, LibertyLink, Innovator, TruFlex, Phytaseed canola, Navigator canola
Maize (<i>Zea mays</i>)	Antibiotic resistance; male sterility; fertility restoration; visual marker; glufosinate herbicide tolerance; modified α -amylase; mannose metabolism; glyphosate herbicide tolerance; coleopteran insect resistance; lepidopteran insect resistance; multiple insect resistance; sulfonyleurea herbicide tolerance; 2,4-D herbicide tolerance, and drought stress tolerance	32138 SPT maintainer, Enogen, Agrisure Duracade, Agrisure Duracade 5122, Agrisure Duracade 5222, Herculex RW, Herculex RW Roundup Ready 2, Optimum GAT, Agrisure GT/CB/LL, Agrisure 3000GT, NaturGard KnockOut, Maximizer, Starlink maize, Enlist maize, Bt Xtra maize, Roundup Ready maize, Agrisure GT, Roundup Ready YieldGard maize, Agrisure RW, YieldGard, MaizeGard, YieldGard VT Triple, YieldGard Rootworm RW, MaxGard, YieldGard Plus, YieldGard Plus with RR, YieldGard RW with RR, Genuity DroughtGard, YieldGard VT Rootworm RR2, Genuity VT Triple Pro, Genuity VT Double Pro, Genuity SmartStax, Power Core, InVigor Maize, YieldGard CB with RR, Liberty Link maize, Herculex I/CB, Optimum Intrasect Xtreme/XTRA, Herculex XTRA/RR/I RR, Optimum TRIssect, Hysyn 101 RR Roundup Ready, and Intacta Roundup Ready 2 Pro
Bean (<i>Phaseolus vulgaris</i>)	Viral disease resistance	N/A
Melon (<i>Cucumis melo</i>)	Delayed ripening/senescence and antibiotic resistance	N/A
Rice (<i>Oryza sativa</i>)	Antibiotic resistance, antiallergenic, lepidopteran insect resistance, and glufosinate herbicide tolerance	BT Shanyou 63, Huahui-1, and Liberty Link rice
Papaya (<i>Carica papaya</i>)	Viral disease resistance and an antibiotic resistance visual marker	Rainbow, SunUp, and Huanong No. 1
Potato (<i>Solanum tuberosum</i>)	Coleopteran insect resistance, antibiotic resistance, modified starch/carbohydrate levels, reduced acrylamide potential, black spot bruise tolerance, and viral disease resistance	Lugovskoi plus, Elizaveta plus, Starch potato, Atlantic New Leaf potato, New Leaf Russet Burbank potato, Innate Russet Burbank potato, Innate G/H potato, Hi-Lite NewLeafY potato, Innate Atlantic potato, New LeafY Russet Burbank potato, New Leaf plus Russet Burbank potato, Shepody NewLeafY potato, Innate Snowden potato

Table 4.1: Summary lists of the genetic modified foods (GMFs) (Zhang et al., 2016a). (cont.)

Scientific Names	GM Traits	Trade Names
Soybean (<i>Glycine max</i>)	Modified oil/fatty acid content; visual antibiotic resistance marker; glufosinate herbicide tolerance; sulfonylurea herbicide tolerance; glyphosate herbicide tolerance; 2,4-D herbicide tolerance; isoxaflutole herbicide tolerance; drought stress tolerance; lepidopteran insect resistance; dicamba herbicide tolerance; and mesotrione herbicide tolerance	Liberty Link soybean, Cultivance, Enlist soybean, Treus, Plenish, Optimum GAT, Roundup Ready soybean, Verdeca HB4 soybean, Intacta Roundup Ready 2 Pro, Vistive Gold, Genuity Roundup Ready 2 Xtend, Genuity Roundup Ready 2 Yield, Herbicide-tolerant soybean line
Tomato (<i>Lycopersicon esculentum</i>)	Delayed ripening/senescence, antibiotic resistance, lepidopteran insect resistance, delayed fruit softening, and viral disease resistance	FLAVR SAVR

GM, Genetic modified.

Characteristics of these GMOs are classified as:

- Improved product quality (i.e., durability and firmness).
- Enhanced pest resistance (i.e., insects and viruses).
- Improved agronomical benefits (i.e., herbicide tolerance) (Lüthy, 1999).

The subject of GMFs is a sensitive issue that divides opinion; some people appreciate their advantages while others believe that GM foods have a negative effect on human health (Dona and Arvanityannis, 2009).

2 Advantages of Genetically Modified Foods

GMFs represent one of the most progressive parts of biotechnology. Benefits of the first generation of GMFs were orientated toward production processes and companies, while the 2nd generation of GMFs offered various advantages and added value for consumers (Zhang et al., 2016b). Producing GMFs (i.e., corn) can help to eliminate chemical pesticide use and reduce the cost of bringing a crop to market (Holst-Jensen, 2009).

Modern biotechnology has focused on techniques to produce viable GMOs for direct consumption; however, one should not forget the possibility of using GMFs as a tool to help solve the hunger problem in developing countries. Drought-resistant crops, nematode-resistant GM potatoes, and herbicide-resistant GM soybeans are now improving the quality of life of millions of people around the world (Zhang et al., 2016b). The main benefits of GMFs are presented later.

2.1 Herbicide Tolerance

The application of herbicides is a necessary part of modern agriculture as unsupervised weeds can lead to crop losses of 20%–60%. The major benefits of growing herbicide-tolerant crops are the increased yields and the reduced costs. Moisture loss and soil erosion can be minimized by nontillage farming (Holst-Jensen, 2009). Watkinson et al. (2000) reported that that weeds can be prevented or eliminated depending on the exact form of management and this would mean that a source of food for birds would be lost. The effects can also be seen on the local flora. For example, bees and butterflies do not have the same efficiency as when GM herbicide-tolerant (GMHT) crops were not employed. Recent studies have shown that bees do not have a preference for GMHT crops, even though there are differences between the quantity and/or quality of the pollen or nectar. Bees and butterflies travel great distances therefore the extent of the damage cannot be accurately calculated (Firbank et al., 2003).

2.2 Insect and Pest Resistance

The ideal plant insecticide gene must (Boulter, 1993):

- Not affect host plant function or metabolic performance in a transgenic plant.
- Be effective against insects, either alone or as part of a system.
- Have broad-spectrum activity against pests, but not affect beneficial insects.
- Not affect animals.
- Not affect people.
- Minimize the buildup of resistant insect populations.

Many accounts of the health, environmental, and economic benefits of insect-resistant transgenic crops have been published (Kennedy, 2008). Insect resistance conferred via expression of a variety of *Bacillus thuringiensis* (*Bt*) delta-endotoxins, is the second most commonly used trait in commercial GM crops, after herbicide resistance (Kennedy, 2008; O'Callaghan et al., 2005). A transgene can produce toxins that are specific to insects that feed on the crop. These genes have been widely used and are already leading to substantial reductions in the use of pesticides and insecticides. Plants expressing endotoxins from *Bt* have been grown on 19% of the global area of transgenic crops and this strategy has been used to produce insect-resistance in crops, such as canola, corn, cotton, maize and potato in both developed and developing countries (Phipps and Park, 2002; Schuler et al., 1998). Major benefits of *Bt*-expressing plants include increased crop yields, reduced insecticide use, reduced fungal toxins levels, and enhanced populations of beneficial insects. To minimize the development of resistance to the *Bt* toxin, field management practices, such as maintenance of refuges are implemented (Engel et al., 2002; Slater et al., 2003). Nowadays, *Bt*-expressing crops also contain one of several Cry proteins for the control of lepidopteran

Table 4.2: *Bt*-protected crops fully approved in the United States (Betz et al., 2000).

Crops	Cry Protein	Pest(s) Controlled
Corn	Cry1Ab	European corn borer, corn earworm, and southwestern corn borer
Corn	Cry1Ac	European corn borer and southwestern corn borer
Potato	Cry3A	Colorado potato beetle
Cotton	Cry3Ac	Tobacco budworm, pink bollworm, and cotton bollworm

or coleopteran insect pests. Table 4.2 summarizes the *Bt*-protected crops fully approved in the United States.

2.3 Disease Resistance

Viruses, fungi, and bacteria can all cause diseases in plants. Plant biologists are working to create plants with genetically engineered resistance to these diseases, as well as resistance to environmental changes. For example, unexpected frost can destroy susceptible seedlings if they are not tolerant to the cold. Therefore an antifreeze gene from coldwater fish has been introduced into plants, such as tobacco and potato to allow these crops to grow in temperatures that would normally kill the seedlings (Godfray et al., 2010; Meyerson and Reaser, 2002; Uzogara, 2000).

Diseases affect the sustainability of a community and animal diseases also affect human health because contamination compromises food safety (i.e., with infectious pathogens or with chemicals used to treat infections), because of zoonotic infections, or because of antibiotic resistance (i.e., the inappropriate use of antibiotics). Diseases can be avoided by various strategies, such as:

1. Ribonucleic acid interference (RNAi); whereby specific short RNA sequences adhere to the RNA of the pathogen, resulting in the destruction of the foreign RNA.
2. Dominant-negative proteins; whereby the introduction of a cell-surface receptor against the pathogen infection blocks disease progression.
3. Ribonucleic acid decoys; where the activity of the pathogen's replication machinery can be destroyed with the expression of RNA sequences that mimic specific sequences within the pathogen.
4. Antibodies; where the transgenic production of antibodies in the host animal may act in an analogous manner to vaccination (Whitelaw and Sang, 2005).

2.4 Temperature Tolerance

High temperatures greatly affect agriculture and the environment. Wang et al. (2003) reported that high temperatures, frequently accompanied by oxidative stress, might cause denaturation of structural and functional proteins. As a consequence these diverse environmental stresses

often activate similar cellular responses and cell signaling pathways, such as the regulation of antioxidants, the accumulation of compatible solutions, and the production of stress proteins. Other studies have shown that small heat-shock proteins in plants are not only expressed in response to high temperatures, but also in response to low temperatures, oxidative stress, salinity, and submergence. It has also been demonstrated that transgenic tobacco plants overexpressing chloroplastic superoxide dismutase have increased resistance to oxidative stress caused by low temperatures and high light (Wang et al., 2003). Whitman (2000) showed that the insertion of an antifreeze gene from coldwater fish in plants, such as potato and tobacco plants enabled them to withstand low temperatures that would normally kill them.

2.5 Salinity Tolerance

As the global population grows more land will need to be used for housing, therefore farmers will be required to grow crops in locations previously unsuited for plant cultivation. Developing plants that can withstand long periods of drought or salinity will enable crops to be grown in formerly inhospitable places (Grieve et al., 2012; Whitman, 2000; Zhu, 2001). Approximately 20% of the global cultivated land and 50% of all irrigated lands are currently affected by salinity. Salinity is measured (in units of electrical conductivity) in saturated soil-paste extracts taken from the root zone of the plant. The advantage of using saturation extracts refers to the field moisture range for most soils. The general effect of salinity is to reduce the growth rate resulting in smaller leaves, fewer leaves, or shorter stature. Moderate salinity produces osmotic effects, ionic stresses, secondary stresses (i.e., oxidative stress), and whole-plant coordination (Chinnusamy and Zhu, 2003; Shannon and Grieve, 1998; Zhu, 2000). Salinity is an increasingly common threat to crop yields (especially in countries where irrigation is essential to agriculture) and is a major abiotic stress in global plant agriculture.

The production of salt-tolerant crops was desired for a long time and the possible methods to increase tolerance have been extensively studied. The evaluation of salt tolerance is made more complex by variations in salt sensitivity during the life cycle of the plant. For example, salinity decreases the grain yield of rice more than the vegetative growth (i.e., germination is resistant to salt). In the case of tomatoes, salinity tolerance at germination is not correlated with the ability to grow under salt stress, or in the case of rice the least three genes represents an important factor in yield under saline conditions. Salt tolerance has also been reported to be a multigene trait and the main characteristics of salt tolerance are represented by a number of subtraits, including an ability to minimize the net accumulation of sodium and chloride ions and to select potassium from a background of high sodium (Flowers, 2004). A separate study investigated the genetics of salinity tolerance in rice using a nine-parent complete diallel cross. The parents were classified as susceptible (IR28, IR29, and MI-48), moderately tolerant (IR4595-4-1-13, IR9884-54-3-1E-P1, and IR10206-29-2-1), and tolerant ("Nona

Bokra,” “Pokkali,” and SR26B). After 19 days growth in salinized culture conditions it was observed that a low Na:K ratio was determined by additive genetic effects. It was concluded that the best offspring had susceptible parents crossed either to moderate or tolerant parents and reciprocal effects among crosses required the use of susceptible male parents in hybridization programs (Gregorio and Senadhira, 1993).

2.6 Nutrition

Malnutrition is a common problem in poor nations. These nations are generally reliant on a single crop, such as rice; however, rice does not contain all the necessary constituents for nourishment. If rice could be genetically manipulated to contain the necessary vitamins and minerals, nutrient deficiencies could be alleviated; for example, blindness is a common problem in poor countries due to a lack of vitamin A (Whitman, 2000). The Institute of Animal Nutrition of the Federal Agricultural Research Centre has performed a few studies using different genetically modified plant (GMP) feeds. Feeds, such as beet sugar and corn were grown with isogenic counterparts in adequate conditions and soybeans and potatoes have been produced by other institutions. Parameters, such as food intake, digestibility, animal health, and the effect on the quality of food from the animals were taken into consideration. Egg laying by hens and quail reproduction were also considered as analysis parameters. The results showed that there was no difference between the modified and unmodified feeds in animals raised for food (even chickens, hens, and quails). Moreover, they used the most sensitive DNA detection methods to identify proteins introduced into crops and established that it does not exist in animal products, chickens, hens, or broilers. The level of security in animals fed modified food was the same as those fed traditional feed (Aulrich et al., 2001; Flachowsky et al., 2005, 2007; Tufarelli et al., 2015).

3 Common Genetically Modified Foods

3.1 Genetically Modified Plants (GMPs)

Several international organizations are investigating the risks associated with GMOs, particularly with respect to their impact on human (or animal) health and the environment. Their objective is to investigate whether the genetic modification could inadvertently increase the toxicity of the recipient plant and alter its nutritional quality, as GMOs are predominantly used in foods or feeds. One of these investigations is specifically looking at the molecular characterization of the genetically modified plants (GMPs) (Hodgson, 2001; Pauwels et al., 2015).

The main techniques used to produce GMPs are “gene guns,” which shoot microscopic particles coated with DNA into the plant cell, or *Agrobacterium tumefaciens*, which

Table 4.3: Content of chlorogenic acid, solanine, total glycoalkaloids, trypsin inhibitors, and chaconine (Novak and Haslberger, 2000).

Antinutrient	Starch Potato		Amylopectin Potato	
	Modified	Control	Modified	Control
Chlorogenic acid	66 $\mu\text{mol}/100\text{ g}$	81 $\mu\text{mol}/100\text{ g}$	—	—
Solanine	94 mg/kg dry weight	98 mg/kg dry weight	50 $\mu\text{g}/\text{g}$ product	64 $\mu\text{g}/\text{g}$ product
Total glycoalkaloids	334 mg/kg dry weight	319 mg/kg dry weight	35 $\mu\text{g}/\text{g}$ product	45 $\mu\text{g}/\text{g}$ product
Trypsin inhibitors	—	—	1.5 mg/g	1.2 mg/g
Chaconine	240 mg/kg dry weight	221 mg/kg dry weight	30 $\mu\text{g}/\text{g}$ product	74 $\mu\text{g}/\text{g}$ product

is a bacteria species that can naturally transfer DNA to plants. These techniques led to global concern regarding human health, therefore they use selectable markers to identify transformed cells, transfer extraneous DNA into the plant genome, and increase the mutation rate in GM compared to non-GM plants (Key et al., 2008). Some examples of widely used GM plants are described later.

3.1.1 Potatoes

Potatoes are GM to improve insect resistance and to enlarge the amylopectin fraction (through alteration of the starch composition). Potatoes contain toxins, antinutrients, solanine, phenols, and alkaloids. If a potato is GM the alkaloid levels must not increase. Table 4.3 summarizes the chlorogenic acid, solanine, total glycoalkaloid, trypsin inhibitor, and chaconine content in GM potatoes. It has been reported that the glycoalkaloid content can vary due to cultivar differences and not because of genetic modifications.

Domingo (2007) compared transgenic potatoes (carrying the *cryI* gene of *Bt* var. *kurstaki* strain HD1) with potatoes treated with the “delta-endotoxin” isolated from the same bacterial strain. Mice were fed with these potatoes before their ileums were extracted. Analysis of the microscopic architecture of enterocytes at the luminal surface showed mitochondria with signs of degeneration and disrupted short microvilli in both groups of mice. Hyperplastic cells were observed in mice fed with the “delta-endotoxin.” (Domingo, 2007).

3.1.2 Maize/Corn

Maize was one of the first innovations of the world for more than 10,000 years (Gewin, 2003). Fernandes et al. (2014) reported that maize is the second most important GM crop and it has the highest number of authorized GM events for food and feed in the European Union. The main goal of GM maize is to enhance insecticide and herbicide resistance (Novak and Haslberger, 2000). S eralini et al. (2007) fed experimental rats with GMOs (transgenic corn MON863) and controls with an equivalent non-GMO diet. They observed a weight decrease in males ($\sim 3.3\%$) and a weight increase in females ($\sim 3.7\%$). They also reported signs of hepatorenal toxicity; in females the triglycerides content grew by

24%–40% and in males the urine phosphorus and sodium excretions decreased by 31%–35%. Therefore GM corn MON863 was not considered to be a safe product. Conversely, [Domingo \(2007\)](#) reported a study where female BN rats and B10A mice were fed with GM corn CBH351, which contained the Cry9C protein derived from *Bt* subspecies *tolworthi*. In this experiment the control mice were fed with isoline corn (non-GM corn). After 13 weeks there was no difference in amino acid, fatty acid, or phytate composition between the animals fed GM or non-GM corn. In addition, no differences in weight were observed and no differences were seen in histological examinations of the spleen, bone marrow, small intestines, liver, or kidney ([Domingo, 2007](#)).

3.1.3 Rice

Rice is one of the main staple foods of the world and it was the first crop species to have its complete genome sequenced ([Lu and Snow, 2005](#); [Singh et al., 2014](#)). [Junhua et al. \(2005\)](#) compared the nutritional composition of GM rice (with cowpea trypsin inhibitor) and parental rice. They showed that the nutrient composition did not vary between these two strains, but some differences in fat content and fatty acid composition were observed ([Table 4.4](#)). The authors concluded that the nutritional composition of this particular GM rice did not change with the insertion of a foreign gene.

[Domingo \(2007\)](#) studied rats and mice fed with rice modified to contain the antiherbicide gene (*BAR*). They reported that there were no adverse effects body weight or histopathological alterations.

3.1.4 Soybeans

Soybeans contain antinutrients and inherent plant isoflavones, which become dangerous to humans when they are associated with food ([Novak and Haslberger, 2000](#)). [Teshima et al. \(2000\)](#) studied the effects of glyphosate-tolerant soybeans, which contain the bacterial 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, on B10A mice and BN rats. The animals were fed with either GM glyphosate-tolerant soybeans or non-GM soybeans. Growth and histopathology of immune-related organs was not different

Table 4.4: Comparison of nutrients in parental rice and GM rice (g/100 g of edible portion) ([Junhua et al., 2005](#)).

Nutrient	Parental Rice	GM Rice
Moisture	11 ± 0.2	11.5 ± 0.7
Protein	8.3 ± 0.6	8.5 ± 1
Lipid	1.04 ± 0.04	0.82 ± 0.04
Fiber	6.6	6.8
Ash	0.8 ± 0.2	0.8 ± 0.2
Carbohydrate	78.9 ± 0.7	78.4 ± 1.5

Table 4.5: Content of α -tomatine, α -chaconine, and α -solanine in modified tomatoes (Novak and Haslberger, 2000).

Sample	α -Tomatine (mg/kg)		α -Chaconine and α -Solanine (mg/kg)	
	Modified	Control	Modified	Control
TGT7	58	74	<5	<5
Other lines	<15	<15	<5	<5
Fresh fruit	<15	<15	<5	<5

between the two groups and the results also indicated that immunotoxic activity was absent and the production of soybean-specific IgE was not detected in the serum of any group (Teshima et al., 2000). Zhu et al. (2004) compared the effects of GM glyphosate-tolerant soybean and conventional soybean in rats and found that there were no differences in the haematological or urinalysis values and there were no adverse effects.

Domingo (2007) reported that this subject should be studied in depth and that the composition of GM soybeans should also be studied in detail as this crop has been used to prepare soymilk and other health food products.

3.1.5 Tomatoes

One of the most successful GMPs has been “Flavr Savr” tomatoes produced by the Calgene (California), in 1992. These tomatoes were GM to express an antisense gene, which allowed an extended shelf life (Chheda and Vernekar, 2014; Zhang et al., 2016a). Dona and Arvanitoyannis (2009) reported stomach erosion and necrosis in rats fed with Flavr Savr GM tomatoes. Tomatoes have the potential to accumulate glycoalkaloids and the principal antinutrient is α -tomatine. The evaluation of α -tomatine in GM tomatoes is important and other factors, such as solanine and chaconine also have to be controlled. Table 4.5 shows the antinutrient values of GM tomato strains, and the results show that the glycoalkaloid levels in the modified tomatoes comply with standards and the genetic modification has not altered the glycoalkaloid levels.

Aumaitre et al. (2002) reported that GM tomatoes expressing *cry1Ab* were safe for rats as no adverse effect were detected after 91 days. Pryme and Lembcke (2003) fed young rats with GM tomatoes expressing *cry1Ab* and observed no difference in body weight or macroscopic organ changes between animals fed GM or control tomatoes.

3.2 Genetically Modified Animals

A GM animal is one whose genetic material has been modified through the use of genetic engineering or recombinant DNA technology (the animal contains foreign DNA either from the same or a different species) (Seney et al., 2015). Savulescu (2013) reported that the term

Table 4.6: Transgenic animals with potential commercial applications (Lievens et al., 2015).

Species	Categories	Transgene	Origin	Effects
Chicken	Livestock	Alv6 envelope glycoprotein	Viral	Disease resistance
		LacZ	Bacterial	Animal health
		Short hairpin RNA	Viral	Disease resistance
	Bioreactor	α -Interferon	Human	Hepatitis treatment
Cattle	Livestock	Lysozyme	Human	Milk composition
		PrP	Knockout	Animal health
		Omega-3	Nematode	Milk composition
	Bioreactor	Lactoferrin	Human	Prophylactic treatment
Carp	Livestock	Growth hormone	Piscine	Growth rate
		Lactoferrin	Human	Disease resistance
Pig	Livestock	Phytase	<i>E. coli</i> , mouse	Feed uptake
		Lysozyme	Human	Piglet survival
		Omega-3	Nematode	Meat composition
	Bioreactor	DAF	Human	Human transplantation
		Fibrinogen	Human	Tissue sealant
		Protein C	Human	Blood coagulation
Rabbit	Bioreactor	Calcitonin	Salmon	Osteoporosis treatment
		Superoxide dismutase	Human	Blood purification
		Growth hormone	Human	HGH insufficiency treatment

“genetically modified animals” refers to transgenic animals, hybrids (created by mixing the sperm of one species with the ovum of another species), and chimeras (created by mixing cells from the embryo of one animal with those of a different species). The Organization for Economic Co-operation and Development and the FAO/WHO decided that the food safety assessment of GM animals must include the molecular characterization of the inserted DNA and the safety of the gene products. A Canadian report also suggested that nutritional analysis (analysis of nutrients and bioactive compounds), the well-being of the transgenic animal, and toxicology should also be included in the safety assessment (Kleter and Kuiper, 2002). GM animals have been possible since the 1980s and since then a wide range of GM animals have been developed for medical research, the creation of disease-resistant species, the production of useful biological substances, human consumption, and xenotransplantation research (Savulescu, 2013; Whitelaw and Sang, 2005). Table 4.6 summarizes some transgenic animals with potential commercial applications.

The production of species that are more resistant to diseases and the production of animals with enhanced meat and milk qualities are the biggest advantages of transgenic animals (Pandey et al., 2016). Transgenic animals are also used to explore gene function, particularly in the context of a specific disease. This method consists of injecting the transgenic construct

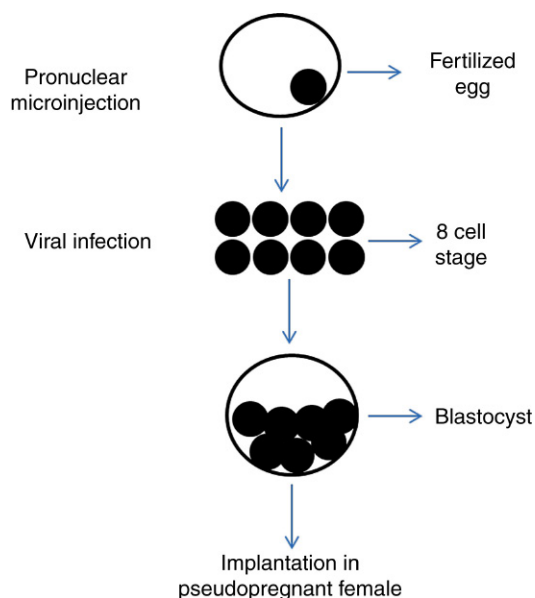


Figure 4.3: The Creation Mechanism of Transgenic Mice.

into the nucleus of a fertilized egg (pronuclear microinjection), allowing the egg to develop to the blastocyst (in vitro), and implanting the egg in a pseudopregnant female (Fig. 4.3) (Seney et al., 2015).

The high availability of suitable animal models to study disease pathophysiology and evaluate novel therapeutic strategies is advantageous for pharmacological studies. Animal models of human hereditary diseases are generally based on mutating the homologous murine genes. In Alzheimer's disease, for example, transgenic mice overexpress the human Alzheimer precursor protein sevenfold. The animals display neurotic changes and congophilic plaques and this mouse line can be used to investigate the role of the β -amyloid (A β) in disease pathogenesis and to test drugs for inhibiting this process (Rudolph and Möhler, 1999). Smith et al. (2005) studied calcium signaling in the triple transgenic mouse model of Alzheimer's disease (3xTg-AD) and observed the intraneuronal accumulation of β -amyloid as an initiating factor in synaptic dysfunction and behavioral deficits. They also reported that the ability of A β to disrupt intracellular calcium homeostasis plays a key role in synaptic dysfunction (Smith et al., 2005).

4 Risks Associated With Genetically Modified Foods

GMFs bring many benefits; however, they are highly controversial and are of concern due to the potential risks they pose to the body (i.e., allergies, toxicity, and genetic hazards). These risks arise from three potential sources: the inserted gene and its expressed proteins; secondary effects or activation of a gene to produce a simultaneous occurrence of several

hereditary characters of the products of gene expression; and the disruption of natural genes in the manipulated organism (Zhang et al., 2016b). There are concerns that transgenic proteins may provoke allergic reactions in sensitive individuals (Moura-Melo et al., 2015).

4.1 Unintended Harm to Other Organisms

The safety of GMFs is a concern as many people consider them to be a threat to human health, animal health, and the environment. One of the main aspects of GMF safety assessment is the evaluation of the unintended effects of exogenous genes (Rischer and Oksman-Caldentey, 2006; Sandmann et al., 2006; Zhang et al., 2012). There are some concerns that the current approach of using targeted analyses to compare the composition of GM crops with their traditional counterparts is biased and doesn't report the unintended effects, which could result (either directly or indirectly) from the genetic modification. The main concerns at present are the unintended effects arising from the application of recombinant DNA techniques in the production of foods. The main effects of GM technologies are listed here:

- Unintended effects refers to the significant difference in the phenotype, response, or composition of the GM plant compared with the parent from which it is derived, and in this case it must be considered as an effect of the target gene. These tests should be done when non-GM and GM counterparts are grown under the same regimes and environments.
- Intended effects of genetic engineering refer to the effects that occur from the introduction of a gene and that fulfill the original objectives of the genetic transformation process.
- Predictable unintended effects refer to the unintended changes that exceed the primary expected effects of introducing the target gene.
- Unpredictable unintended effects refer to the changes falling outside the present level of understanding. The last two effects may be unimportant in terms of product safety, but they must be considered when assessing risk (Cellini et al., 2004).

Ladics et al. (2015) reported that semidifferentiated plant tissues cultured in vitro are critical for most plant transformation methods; however, in vitro culture induces somaclonal variation (SCV), which represents an unintended effect. Several studies have attempted to reduce the severity of SCV and the most effective way to eliminate it in barley was to backcross transgenic plants to plants without SCV and perform selection at each generation based only on the presence of the transgene. In the case of the barley cultivar, Conlon, a single backcross recovered the majority of yield loss caused by SCV in transgenic Conlon-derived lines. The yield loss was ~6% in the backcross-derived lines compared with 31% in the primary transgenic lines (Ladics et al., 2015). A laboratory study found that pollen from *Bt* corn caused high mortality rates in monarch butterfly caterpillars; however, these animals eat milkweed plants and not corn. This raised the possibility that pollen from *Bt* corn could be blown by the wind and hit the milkweed plants in neighboring fields and if caterpillars eat this pollen they could perish. Although this study was not conducted in a natural field the authors drew this conclusion. The *Bt* toxins do kill some larvae species but it is not

Table 4.7: Unintended effects by phenotype selection or investigation of defined constituents (Cellini et al., 2004).

Host Plant	Traits	Unintended Effects
Potato	Expression of bacterial levansucrase	Adverse tuber tissue perturbations
Soybean	Expression of glyphosate resistance	Splitting stems and yield reduction (~40%) at high soil temperatures (45°C)
Wheat	Expression of glucose oxidase	Phytotoxicity
Rice	Expression of soybean glycinin	Increased vitamin-B6 content (+50%)
Potato	Expression of yeast invertase	Reduced glycoalkaloid content
Rice	Expression of carotenoid biosynthetic pathway	Formation of unexpected carotenoid derivatives
Canola	Overexpression of phytoene synthase	Multiple metabolic changes

possible to produce a *Bt* toxin that eliminates damaging crops without affecting other insects (Whitman, 2000). Table 4.7 presents some of the unintended effects by phenotype selection or investigation of defined constituents.

4.2 Reduced Effectiveness of Pesticides

Pest control using environmentally harmful chemicals can be reduced with the help of insect-resistant and herbicide-tolerant transgenic crops (Qaim, 2009; Qaim and Zilberman, 2003). Some studies have calculated the impact of herbicide- and insect-resistant crops on pesticide use per hectare, using rapidly spreading glyphosate-resistant weeds or overall pesticide use as endpoints. One model quantified (by crop and by year) the impacts of herbicide-resistant corn, cotton, soybeans, *Bt* corn (targeting the European corn borer), *Bt* cotton (for lepidopteron insects), and *Bt* corn (for corn rootworms) from 1996 to 2011. In the United States the herbicide-resistant crop technology led to an increase of 527 million pound, the *Bt* crops reduced insecticide applications about 123 million pounds, and the pesticide use increased about 404 million pounds between 1996 and 2011. Benbrook (2012) reported that pesticide use in the United States was reduced in the first 6 years following the commercial use of GM crops. One of the major advantages of using *Bt*-protected plants (since 1996) is the reduction in chemical insecticide use. In 1998 the chemical-insecticide control of bollworm and budworm was reduced by approximately 2 million pounds in six key cotton-producing states compared to the use in 1995 (Betz et al., 2000). However, many people are concerned that insects will become resistant to *Bt* as was seen with mosquitoes that developed resistance to the pesticide DDT (Whitman, 2000).

Lusk et al. (2004) reported that GM crops reduced pesticide use by 4.4%. Pesticide use threatens the environment, health, and the survival of birds, insects, and fish; therefore researchers have tried to reduce this problem. Many studies maintain that pesticides reduce

species diversity in the animal kingdom and contribute to population decline of plants and animals by reducing food supplies, destroying habitats, and impairing reproduction. A special protein found in vegetable oil was identified and derived from GM crops. This special protein allows the plant to be resistant to certain insects (Lusk et al., 2004) and its use allows farmers to reduce pesticide use.

4.3 Gene Transfer to Nontarget Species

One major concern is that the crops engineered for herbicide tolerance will crossbreed with weeds and this may result in the production of herbicide-tolerant weeds. (Whitman, 2000).

Dale et al. (2002) reported that the transfer of genes from GM crops (via animal feed) doesn't represent a problem because survival through the entire gastrointestinal tract isn't possible. For example, transgenic DNA from glyphosate tolerant soybeans was completely degraded in 15 s when incubated in simulated gastric fluid. Even if the DNA can persist in the gut long enough to be taken up by bacteria, further studies must be performed to establish if gene transfer to other gut microbes is possible (Dale et al., 2002).

The most popular technology of gene transfer to plants using *A. tumefaciens* is based on horizontal gene transfer (HGT); however, the mechanism for HGT from plants is not well understood. In theory, HGT of plant DNA requires: availability (during growth the introduced transgenes should be available for transfer and the free DNA should have a length sufficient for uptake); uptake (bacterial should be competent for DNA uptake); and establishment (incorporation, maintenance, and use of the incoming DNA) (Conner et al., 2003; Eastham and Sweet, 2002).

4.4 Human Health Related Risks

The major problem of GMFs in relation to human health is allergenicity. Sensitive people who come into contact with GMFs could have a serious allergic reaction (Whitman, 2000) and unlabeled GMFs may cause anaphylaxis (Pusztai et al., 2003). It was reported that many children have developed allergies to peanuts and other foods. The Codex Committee on Food Labelling produced a list where the most common allergenic foods associated with IgE-mediated reactions (i.e., fish, nuts, crustacea, eggs, milk, soybeans, wheat, and peanuts) are found. The world is currently facing two situations:

1. When a food contains a gene derived from a known allergenic source: Before deciding if the GMF is safe for humans the food must go through two steps. The first step establishes sequence homology, and if the food is found to contain an allergen it is considered allergenic and is emitted from future testing. The subsequent step for assessment of

Table 4.8: Clinical patterns of immune-mediated adverse reaction to foods (Metcalf, 2003).

Allergen	Adverse Reaction
IgE mediated (immediate)	Urticaria/angioedema, rhinoconjunctivitis, asthma, anaphylaxis, colic, vomiting, and diarrhea
Non-IgE mediated (delayed)	Food-induced enterocolitis syndromes
Controversial	Irritable bowel syndrome, migraine, and headaches
Overlap	Allergic eosinophilic gastroenteritis and atopic dermatitis

potential allergenicity involves testing the expressed protein with sera from patients allergic to the source material.

- When a food contains a gene derived from a source not known to be allergenic: The product must go through four steps. The first step establishes sequence homology to known allergens; the second step uses targeted serum screening for cross-reactivity with sera from patients allergic to materials that are broadly related to the source material for the gene; the third step tests the pepsin resistance; and the final step tests the immunogenicity in animal models (Metcalf et al., 1996; Taylor, 1997; Taylor and Hefle, 2001).

Table 4.8 summarizes some of the clinical patterns of immune-mediated adverse reactions to foods.

It is true that if a protein is derived from a source with a history of allergenicity then the GM crop will be allergenic; however, when a gene is derived from a source that is without allergenicity the GM crop will not be allergenic (Goodman et al., 2008; Pusztai et al., 2003).

5 Economic Concerns

Developing countries, such as Argentina, China, Brazil, South Africa, and India have approved GM crops for commercial release, but countries, such as Latin America are against herbicide-tolerant soybeans (Pandey et al., 2016). Bringing a GMF to market is a very difficult process because it requires a lot of time and money (Akumo et al., 2013). Many GM plants have been patented and a major concern of agribusiness is patent infringement. Third world countries and small farmers are worried about the rising prices of these new plant varieties. Whitman (2000) reported that rising prices could create major problems for many of farmers. However, on a positive note the Rockefeller Foundation have offered their products to impoverished nations at reduced costs. Zhang et al. (2016a) reported that the global increase in farm income owing to GMFs was approximately US \$116 billion in 2006–12, almost triple that of the last decade. It was also reported that the advanced genetics and resistance to pests and weeds of GMFs resulted in an economic gain of 42%. Another advantage was the decreased cost of production.

As mentioned previously, patent infringement is another problem. One proposed solution is to place a “suicide gene” into the GM plants. These plants would therefore only be viable for one growing season and produce sterile seeds that do not germinate and the farmers would need to buy a new supply of seeds every year. However, this would be detrimental for those farmers who cannot afford to buy seed each year (Whitman, 2000).

Gurău and Ranchhod (2016) compared the advantages and disadvantages of GMFs and traditional foods:

- GMFs are cheaper than traditional foods if the new plant/animal has enhanced productivity/resistance to pest and diseases.
- GMFs can provide higher additional value than traditional foods.
- GMFs can be used to deliver therapeutic substances, which may replace traditional medications or vaccines.
- GMFs can be used to improve the quality of the environment by reducing pollution.
- GMFs may represent a solution to the global food crisis.

The insecticide-reducing and yield-increasing effects of GM crops are highest in countries, such as India and Argentina. In the case of *Bt* cotton, chemical insecticides are used in Argentina and insect pests are not effectively controlled, but in India insecticide use in conventional cotton is much higher. Therefore the insecticide resistance, insecticide quality, and the correct choice of products are important. Similar effects were observed for *Bt* maize. For example, it is used mainly against the European corn borer in the United States, as this is not controlled by chemical means. In South Africa the pest pressure is more severe than it is in temperate climates and the mean yield effects are higher. Average yield gains on *Bt* plots of smallholder farmers were 32% in South Africa and 34% in the Philippines. The effects of *Bt* rice on yield were relatively small in China while the introduction of *Bt* eggplant in India had significant insecticide and yield effects. These data are presented in Table 4.9.

Table 4.9: The agronomic and economic effects of *Bt* crops in farms (Qaim, 2009).

<i>Bt</i> Crops	Countries	Insecticide Reduction (%)	Increase in Effective Yield (%)	Increase in Gross Margin (US \$/ha)
<i>Bt</i> cotton	United States	36	10	58
	South Africa	33	22	91
	Mexico	77	9	295
	India	41	37	135
	China	65	24	470
	Argentina	47	33	23
<i>Bt</i> maize	United States	8	5	12
	South Africa	10	11	42
	Philippines	5	34	53
	Spain	63	6	70
	Argentina	0	9	20

Another study reported that the use of pesticides on GM cropped areas has reduced by 9% since 1996 and the environmental impact decreased by 17.9%. In 2010 the total carbon dioxide emission savings associated with GM crop adoption were equal to the removal of 8.6 million cars from the roads (Garcia-Yi et al., 2014).

6 Conclusions and Perspectives

GMFs have the potential to solve many of the world's problems; however, many challenges still exist, especially in the areas of safety testing. Even if GM has increased the production of some crops, the technology has been met with some challenges, such as the production systems, especially in many developing countries.

In the near future we expect an increased production of GMOs for use in foods, drugs, and environmental-related applications. They are also likely to be important for the production of fruit and nut trees that yield years earlier and plants that produce new biodegradable plastics with unique properties. More studies regarding the potential health risks of GMFs will also be important in the future. Methods and concepts are needed to probe into the compositional, nutritional, toxicological, and metabolic differences between GM and conventional crops (Bawa and Anilakumar, 2013). The future testing technologies are expected to be faster, cheaper, sufficiently specific, and quantitative and must allow multiplexing and quantitation (Holst-Jensen, 2009; Pandey et al., 2010) in order to offer a wide and efficient perspective on the utilization of novel GMFs.

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Influence of Feed From Genetically Modified Plants on the Composition and Quality of Foods of Animal Origin

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1 Introduction

The composition and quality of foods of animal origin may be substantially influenced by animal feeding. Enhancing the nutritional content of foods of animal origin provides an opportunity to increase nutrient levels in the human diet, which is especially important for those who are marginally or substantially malnourished. It is well known that fatty acids, minerals, and vitamins are transferred from feed into foods of animal origin (Table 5.1); however, there are some differences between ruminants (e.g., cattle and sheep) and nonruminants (e.g., pigs and poultry) due to the influence of microbiota in the rumen (i.e., the fermentation processes differ, especially for unsaturated fatty acids).

Some genetically modified plants (GMPs) can influence the composition and organoleptic qualities of foods of animal origin, such as the distribution of fat and lean meat in the carcass; fat grade and fatty-acid composition; mineral and vitamin concentration in the body, organs, tissues, milk and eggs; yolk and meat color; and other quality measurements, such as tenderness and flavor. Specialized studies/measurements are necessary to assess the influence of GM feeds on the composition/quality of foods of animal origin (EFSA, 2012; Flachowsky et al., 2012; ILSI, 2007) and Table 5.2 shows some endpoints used in these analyses.

In addition to recent books (Flachowsky, 2013; NASEM, 2016; Watson and Preedy, 2015) and review papers (Flachowsky and Reuter, 2017; JRC, 2016; Smyth et al., 2015; The Royal Society, 2016; van Eenennaam and Young, 2014), the objective of this chapter is to summarize the current knowledge of GMP feeds and their coproducts, and their effect on the composition and quality of foods of animal origin [i.e., meat, milk, eggs, fish, and other foods (Sections 3 and 4)]. The present global food situation will also be analyzed and the future challenges and trends will be discussed (Section 2).

Table 5.1: The influence of animal nutrition on selected nutrients in foods of animal origin (Flachowsky, 2009).

Nutrients	Milk	Meat ^a	Eggs
Protein/amino acids	(+)	–	–
Fat/fatty acids	+++	++	++
Major elements			
Calcium	–	–	–
Phosphorus	–	–	–
Magnesium	–	–	–
Trace elements			
Copper	(+)	(Liver: +++)	(+)
Iodine	+++	+	+++
Selenium	++	++	++
Zinc	+	+	+
Vitamins			
A	(+)	(Liver: +++)	+
D	+	+	++
E	(+)	(+)	+++
B-vitamins	(+) (If rumen protected)	– to +	– to ++

+++, Very strong influence, the transfer of the supplement into food >10%; ++, strong influence possible, the transfer is 5%–10%; +, moderate influence, the transfer is 1%–5%; (+), small influence, the transfer is <1%; –, no influence, there is no transfer from feed into food.

^aDifferences between meat of ruminants and nonruminants.

Table 5.2: Proposal of endpoints for comparative analysis of foods of animal origin from GM animals and animals fed with 2nd generation GMP feed (EFSA, 2012).

Group of GM Animals	Mammals	Birds	Aquaculture (e.g., Fish and Mollusks)	Insects (Honeybees)
Samples from the animal body	Tissues - Meat and muscle (i.e., longissimus dorsi and biceps femoris) - Body fat - Blood - Organs (i.e., liver, kidney, spleen, and brain) - Residual body parts (e.g., meat and bone meal as feed)	Tissues - Meat and muscle (breast and thigh) - Abdominal fat - Blood - Organs (i.e., liver, kidney, and spleen) - Residual body parts (e.g., animal body meal as feed)	Edible fractions (e.g., fillet) Residual body parts (e.g., fish meal as feed)	–
Food/feed produced by animals	Milk	Eggs	Caviar	Honey

Challenges for plant breeding in the future will likely include plant tolerance to biotic and abiotic stresses (i.e., drought and soil salinity) and the production of plants that are able to utilize carbon dioxide (CO₂) and other nutrients more efficiently. Biofortification of plants may be important to enable plant breeders to avoid and overcome human micronutrient deficiencies in many regions of the world.

New powerful genome editing technologies, such as clustered regularly interspersed short palindromic repeats (CRISPR) may also contribute to the production of more efficient plants.

2 Global Food Situation

Feed is a key element in animal husbandry and the production of foods of animal origin. Feeds can be defined as edible materials that are consumed by animals and contribute energy and/or nutrients (i.e., proteins/amino acids, fatty acids, minerals, and vitamins) to the animal's diet. Feed costs amount to 40%–70% of the total costs of animal production and feed production consumes high amounts of limited natural resources (i.e., water, land area, fuel, fertilizers, and pesticides). Therefore, feed efficiency, also known as feed conversion (the amount of feed per product of animal origin, such as milk, eggs, meat, or fish), is one of the most important parameters to assess feed utilization.

An increasing population size and a higher demand for feed and food are associated with a growing demand for limited natural resources and elevated emissions of greenhouse gases (GHG), such as carbon dioxide, methane (CH₄), nitrous oxide (laughing gas; N₂O), and other substances (e.g., N, P, and trace elements) (Malik et al., 2015). The FAO (2009a,b) reported that the global population will increase from 7 billion to over 9 billion by 2050, but the increase in the output of foods of animal origin is estimated to only be ~70% (Alexandratos and Bruinsma, 2013; HLPE, 2014). These changes characterize the global situation (Ash et al., 2010; Delgado, 2003; FAO, 2006, 2009a,b, 2010; Godfray et al., 2010; Guillou and Matheron, 2014; IPCC, 2006; Vermeulen et al., 2012). The so-called “triple burden” of malnutrition [undernutrition, micronutrient deficiencies (e.g., iron, iodine, and vitamin A), and overnutrition] is still recognized as a serious and intractable human problem (HLPE, 2014; Thompson and Amoroso, 2014). The recent FAO estimates (FAO, 2014) indicate that ~805 million people are chronically undernourished, which amounts to 11.3% of the global population. In addition, many more individuals suffer from micronutrient deficiencies, and foods of animal origin, also called animal source foods (Neumann et al., 2014), may help to overcome these deficiencies.

The energy and protein conversion efficiency from feed into animal-origin foods is low and varies from 3% in beef (energy) to 40% in dairy (energy) and chickens (protein) (Cassidy et al., 2013; Smil, 2000). In the United States, between 67% (energy) and 80% (protein) of crops are used as animal feeds (Cassidy et al., 2013).

Table 5.3: Global intake of milk, meat, eggs, and protein of animal origin per capita and year and portion (%) of total protein intake (global minimum-values, maximum-values, and averages, as well as German values for comparison; kg/capita and year: data base 2005; [FAO, 2009a](#)).

Foods	Global Minimum	Global Average	Global Maximum	German Average
Milk (kg/year)	1.3 (Congo)	82.1	367.7 (Sweden)	248.7
Meat ^a (kg/year)	3.1 (Bangladesh)	41.2	142.5 (Luxembourg)	83.3
Eggs (kg/year)	0.1 (Congo)	9.0	20.2 (China)	11.8
Edible protein of animal origin (g/capita and day)	1.7 (Burundi)	23.9	69.0 (USA)	52.8
Portion of animal protein (% of total protein intake per capita)	4.0 (Burundi)	27.9	59.5 (USA)	53.7

^aProbably empty body weight (meat plus bones).

These developments and complex connections present the following question: “is there any need for foods of animal origin in human nutrition?” Vegans aptly demonstrate that foods of animal origin are not essential if the human diet contains, or is supplemented with, essential nutrients; however, the consumption of meat, fish, milk, and eggs may contribute to meeting amino acid ([D’Mello, 2011](#); [Neumann et al., 2014](#); [Pillai and Kurpad, 2011](#); [Smith et al., 2013](#); [Thompson and Amoroso, 2014](#); [WHO, 2007](#)) and trace nutrient (i.e., Ca, P, Zn, Fe, I, Se, vitamins A, E, B₁₂) requirements. This is especially important for children and juveniles, as well as pregnant and lactating women ([Wennemer et al., 2006](#)). Human nutritionists have recommended that one-third of the daily protein requirement (0.66–1 g/kg of body weight) should be met by proteins of animal origin ([Jackson, 2007](#); [Rand et al., 2003](#); [Waterlow, 1999](#); [WHO, 2007](#)), therefore approximately 20 g of the daily intake of protein (60 g) should be of animal origin. The current average consumption of animal proteins (without fish) is approximately 24 g/capita and day, ranging from 1.7 g in Burundi to 69 g in the United States ([Table 5.3](#)). Overcoming this imbalance is likely to be a challenge in the future ([Smith et al., 2013](#)). Meat, milk, and eggs provide ~13% of the energy and 28% of the protein consumed globally, although these estimates increase to 20 and 40%, respectively, in so-called developed countries ([FAO, 2009a](#)). It is difficult to assess protein intake from fish and other animal protein sources (e.g., insects), although [Avadi and Fréon \(2013\)](#) estimate that half of the world’s population consume at least 15% of their animal protein from aquaculture.

Foods of animal origin are also consumed because they contain high levels of nutrients and for their “enjoyment value.” These foods are also considered to be indicators of the standard of living in many regions of the world. Eating foods of animal origin, especially meat, is not only a reflection of nutritional needs, but it is also determined by taste, odor, and texture, as well as by geographical area, culture, ethics, and wealth. An increased demand for animal-origin foods is also driven by increased incomes ([HLPE, 2014](#); [Kastner et al., 2012](#); [Keyzer](#)

et al., 2005; Tilman et al., 2011) and the want to imitate the “western style of life.” Many developing countries continue to consume more animal products than they produce, therefore these countries will continue to drive the global demand for agricultural products, including foods of animal origin (Guyomard et al., 2013). Higher volumes of animal-origin foods requires higher plant yields (Fischer et al., 2014), and/or increased feed production areas (Fedoroff et al., 2010; Flachowsky et al., 2013, 2017; Gerbens-Leenes and Nonhebel, 2002; Wirsenius et al., 2010), more animals, and/or higher animal yields, and increased agricultural trade. Therefore, the definition of agricultural yield, and agriculture in general, should be changed “from tonnes to people nourished per hectare” (Cassidy et al., 2013; Kastner et al., 2012) and many are calling for the introduction of more sustainable animal agriculture (FAO, 2014; Kebreab, 2013; SAFA, 2013).

Conversely, changing eating patterns (Guyomard et al., 2012) and eating less or no livestock products (especially meat) could be a solution to reduce the environmental impact of animal agriculture (Baroni et al., 2007; Pimentel and Pimentel, 2003; Reijnders and Soret, 2003) and reduce the per capita land requirements (Peters et al., 2007).

The current situation will continue as the population continues to increase, severe constraints are placed on land and water, the demand for food and biofuel increases, and climate change worsens (Malik et al., 2015). There will be strong competition for arable land (Flachowsky et al., 2017); nonrenewable resources, such as fossil carbon sources; water (Deikman et al., 2012; Hoekstra and Champaign, 2007; Renault and Wallender, 2000; Schlink et al., 2010); minerals (i.e., phosphorus; Hall and Hall, 1984; Scholz and Wellmer, 2013); feed, food, fuel, fiber, flower, and fun; (6 F’s-concept; Aerts, 2012); areas for settlements; and natural protected areas.

Plant breeding can be considered as the starting point of the food chain as shown in the simple model in Fig. 5.1 (Flachowsky et al., 2012; Ruane, 2013; The Royal Society, 2009). Traditional breeding, as well as “green” biotechnology or green chemistry may change the composition and nutritive value of feed plants (Guillou and Matheron, 2014; Harvie, 2015; Husaini and Tuteja, 2013).

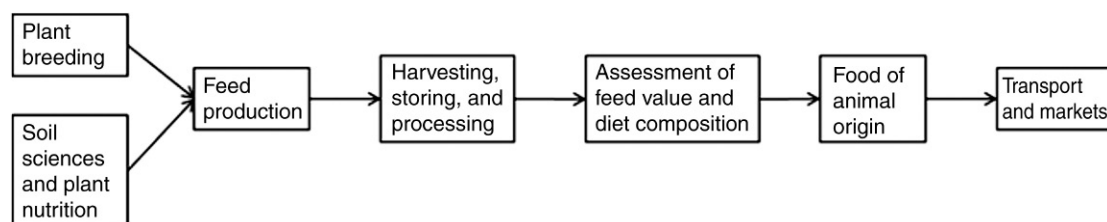


Figure 5.1: Plant breeding, Feed Production, and Feed Science as Key Elements of Production of Foods of Animal Origin in the Human Food Chain.

There is currently a contradictory public and philosophical discussion (Tsegay and Tamiru, 2014) regarding the various methods of plant breeding (i.e., the pros and cons of conventional breeding versus genetic engineering). The global area of GMP cultivation increased from 1.6 million ha in 1996 to 181.5 million ha in 2014 (James, 2015) and reduced slightly to 179.7 million ha in 2015 (James, 2016). This equates to ~12% of global arable land being cultivated with GMPs (primarily soybean, maize, cotton, and rapeseed), which are mainly plants of the so-called first generation (e.g., those with increased pest resistance and herbicide tolerance but without substantial changes in their composition and nutritive value). Many short-term (Flachowsky, 2013; van Eenennaam and Young, 2014; Watson and Preedy, 2015) and long-term feeding studies (Halle and Flachowsky, 2014; Riccio et al., 2013) using food-producing animals have been performed and they demonstrate the equivalence of feeds and coproducts from first-generation GMPs. Animal health and the composition and quality of animal-origin foods were not significantly influenced by GMP feeds. Additional goals of plant breeders include improving the composition and nutritive value of GMPs (e.g., to increase the content of amino acids, vitamins, and enzymes, which is also called biofortification; second-generation GMPs) and reducing the content of undesirable ingredients (i.e., lignin, phytates, and glucosinolates). Herbicide tolerance and pesticide resistance may be advantageous for producers and farmers, while biofortification may increase the content of various nutrients (e.g., fatty acids, vitamins, and trace elements) in foods of animal origin and may contribute to improved human nutrition in some countries (Gangashetty et al., 2016; Smyth et al., 2015, and Table 5.1). Conversely, biofortification for animal nutrition is not urgent as many feed additives are available to complete animal rations (Flachowsky and Meyer, 2015).

One of the present objectives of GMP studies is to equip plants with more than one changed property, and these plants are called stacked events. Maize is the crop with the most commercial stacks, with 96 events currently available (Parisi et al., 2016); six GM transformation events have already been combined in commercial GM maize plants producing 41 double, 35 triple, 13 quadruple, 6 quintuple, and 1 sextuple combinations (Parisi et al., 2016).

Breeding technologies (traditional or GM), novel techniques [e.g., zinc finger endonucleases; clustered regularly interspaced short palindromic repeats (CRISPR)-associated plants, (Irmer, 2016; Quetier, 2016; Samanta et al., 2016; Song et al., 2016); and transcription activator-like effector nucleases (TALENs), (Schaart et al., 2016)], and alternative methods (Lusser et al., 2012) may contribute to solving important global problems, such as the efficient use of limited global resources (e.g., water, arable land, fuel, and minerals) or adaption to climate change and the lowering of global GHG emissions. Increased research activities and political willingness are necessary for the sustainable utilization of limited natural resources and for the improved use of unlimited resources (i.e., sunlight and energy, CO₂, N₂, and the global gene pool). Conventional plant breeding may fulfil some of these

objectives; however, genetic modification is likely to be faster and more precise (Tester and Langridge, 2010). Increased research in this field is also necessary, and “yes or no” are not acceptable answers to the question “GMP or no GMP?,” as all methods of plant breeding that contribute to a more efficient production of high and stable yields of biomass should be used/combined.

Challenges of plant breeding in the future are likely to include the production of plants that are tolerant to biotic and abiotic stresses (i.e., drought and soil salinity), and the production of plants that are more efficient at utilizing CO₂ and other nutrients. Biofortification of plants may also be an important objective of plant breeders as it may help to avoid and overcome human micronutrient deficiencies in many regions of the world. New powerful genome editing technologies, such as CRISPR may contribute to the production of more efficient plants.

3 Feeding Studies With GM Plants of the 1st Generation

GMPs of the 1st generation are characterized by their substantial equivalence (i.e., plants with input traits), which means that their composition does not differ from their isogenic counterparts [with the exception of the transgenic or recombinant DNA fragment(s) and the newly expressed protein(s)]. Therefore, feeds from first-generation transgenic plants do not influence the composition and quality of foods of animal origin. FASS (2016) summarized references pertaining to transgenic DNA and protein and livestock products, such as meat, milk, and eggs.

3.1 Recombinant DNA and Newly Expressed Proteins

Plant recombinant DNA is mainly degraded during ensiling (Aulrich et al., 2004; Hupfer et al., 1999), feed processing (Alexander et al., 2002; Berger et al., 2003a; Chiter et al., 2000; Gawienowski et al., 1999), or in the digestive tract of animals (Alexander et al., 2004, 2007; Einspanier, 2013; Einspanier et al., 2004; Wiedemann et al., 2006).

It is possible for gene fragments to enter the intestinal epithelium and be absorbed by the host organism. Low-copy DNA (e.g., recombinant DNA) has rarely been detected, while multicopy DNA (endogenous DNA; e.g., Rubisco DNA) has been detected in several body samples (Sanden et al., 2011). Many authors have failed to find recombinant DNA fragments in animal body samples, milk, and eggs (De Giacomo et al., 2016; Einspanier, 2013; Einspanier et al., 2001; Furgal-Dierzuk et al., 2014, 2015; Guertler et al., 2009; Morera et al., 2016; Reuter and Aulrich, 2003; Walsh et al., 2011); however, some (mainly Italian) authors (Chainark et al., 2008; Mazza et al., 2005; Ran et al., 2009; Sharma et al., 2006; Tudisco et al., 2006, 2010, 2015) amplified recombinant DNA fragments from animal tissues and milk. However, some of Tudisco’s studies (Tudisco et al., 2010, 2015) have received scientific criticism due to their methodical weaknesses (Abbott, 2016).

Similar studies have also been performed for newly expressed proteins. Such proteins are degraded during feed processing/conservation, and in the digestive tract, as demonstrated during in vitro studies and in digestion and feeding experiments (Buzoianu, 2011; Calsamiglia et al., 2007; Gruber et al., 2011; Guertler et al., 2008; Li et al., 2015; Lutz et al., 2005; Paul et al., 2010; Scheideler et al., 2008; Walsh et al., 2012; Wiedemann et al., 2006). Small protein fragments (e.g., peptides) may also pass the intestinal epithelium and enter the host organism.

Some authors have suggested that foods of animal origin should be labelled if the animals have consumed GM feed (Antoniou et al., 2012; Seralini et al., 2011); however, at present these wishes cannot be scientifically justified due to the inconsistencies in detecting recombinant DNA and proteins in animal tissues, milk, and eggs.

Einspanier (2013) summarized the results concerning recombinant DNA and proteins in animals:

- Fragmented feed DNA and proteins are present in the intestinal tract. Feed DNA fragments may be transferred into the tissues of animals during natural processes.
- The presence of feed DNA fragments in animal tissues does not represent a safety risk to the animal or the consumer.
- When gene fragments from ingested DNA are found in organs, these foreign DNA fragments do not possess biological function and will not account for apparent effects in the animal, nor have they been found to be integrated in the animal genome.
- When reviewing all available data, there is no scientific evidence that milk, meat, or eggs derived from animals fed with GM feed are less safe for the consumer than those produced with conventional feed.

3.2 Product Composition and Quality

Many studies have compared the quality of livestock products obtained from animals fed with first-generation GMP feeds or their isogenic counterparts. Some results of the early studies are exemplarily demonstrated in Tables 5.4–5.12.

Milk composition (e.g., fat, protein, and lactose) and milk quality from dairy cattle were not influenced by GMP feed (first generation), as demonstrated in Table 5.4. Similar results were also reported in a separate long-term study (Table 5.5) where the authors found no significant influence on milk composition during a 25 month experimental period. Further detailed analysis of milk from cows fed with high portions of isogenic or *Bacillus thuringiensis* (Bt) maize did not show any significant differences between the groups (Table 5.6).

Some authors (Aulrich et al., 2001; Berger et al., 2003a,b; Erickson et al., 2003) have reported that the inclusion of first-generation GMPs in the diets of beef cattle did not affect body composition, marbling scores, fat depth, longissimus dorsi area, or yield grade

Table 5.4: Influence of feeding maize silage and maize grain from isogenic and *Bt* maize on food intake, milk production, and milk composition of dairy cows (average of two experiments; 6 or 8 cows per treatment; duration of exp.: 63 days; maize portion in ration; Exp. 1: 41.8% of DM; maize silage; 34.1% of cracked maize; exp. 2: 59.6% maize silage, 19.9% cracked maize; [Donkin et al., 2003](#)).

Parameters	Control Maize	<i>Bt</i> Maize
Dry matter intake (kg/day)	24.3	24.1
Milk yield (kg FCM/day)	33.2	33.2
Milk fat (%)	3.60	3.67
Milk protein (%)	3.10	3.14
Milk lactose (%)	4.62	4.64

Table 5.5: Milk composition of the 1st and 2nd lactation of a long-term feeding study with dairy cows [$n = 18$ per treatment, 25 months with *Bt*-maize (MON810), 63% of roughage, 41% of concentrate from maize, [Spiekens et al., 2009](#)]^a.

Lactation of Experiment	1st Lactation		P Value	2nd Lactation		P Value
Parameters	Isogenic	Transgenic		Isogenic	Transgenic	
Milk yield (kg/day)	23.9	23.7	0.566	29.2	28.8	0.419
Milk fat (%)	3.95	4.03	0.015	3.75	3.86	0.055
Milk protein (%)	3.62	3.71	<0.001	3.59	3.56	0.299
Milk lactose (%)	4.83	4.82	0.155	4.74	4.80	0.006
Milk urea (mg/L)	164	181	0.001	180	175	0.523
Somatic cell count (1000 mL ⁻¹)	157	205	0.073	241	220	0.754

^aNo fragments of Cry1Ab DNA were detected in blood, milk, feces, and urine of cows; traces of Cry1Ab protein were detected in feces, but not in blood, milk, or urine ([Guertler et al., 2009](#)).

([Table 5.7](#)). [Erickson et al. \(2003\)](#) fed high portions of glyphosate-tolerant maize (GA21, NK603) to feedlot steers and measured meat parameters in three separate studies. They found no difference between animals fed with transgenic maize, its isogenic counterpart, or two commercial lines ([Table 5.8](#)).

GMP feeding studies in small ruminants produced similar results to those described in lactating cows, and bulls/steers. [Stanford et al. \(2003\)](#) fed transgenic (RR) canola to lambs (21.5–45 kg body weight) and their results were compared against lambs fed with either the parenteral line or two commercial lines. The inclusion level of canola in the experimental diet

Table 5.6: Selected parameters of milk composition, protein fractions, and fatty-acid proportion in milk of dairy cows fed conventional or *Bt*176 maize (12 cows per treatment; 70% of DM maize silage; [Barriere et al., 2001](#)).

Parameters	Isogenic Control	Transgenic Maize (<i>Bt</i> 176)
Milk yield (kg/day)	31.5	31.8
Milk fat (%)	3.69	3.68
Milk protein (%)	3.20	3.18
Total caseins (g/kg)	24.9	24.9
α -Lactalbumin (g/kg)	1.12	1.16
β -Lactoglobulin (g/kg)	3.86	3.90
Curd yield (%)	15.2	15.0
Fatty acids (% of total)		
C ₄ -C ₁₂	14.3	15.2
Total C ₁₄	13.5	14.0
Total C ₁₆	41.1	41.0
C _{18:0}	8.8	8.5
C _{18:1}	16.5	15.9
C _{18:2}	2.1	2.1

Table 5.7: Influence of corn rootworm protected maize (MON863) on slaughtering data of feedlot steers (initial body weight: 457 kg; duration of experiment: 102 days; [Berger et al., 2003b](#)).

Parameters	Commercial Control RX 740	Commercial Control DK647	Isogenic Control RX670	Transgenic Maize MON863
Body weight gain (kg/day)	1.39	1.49	1.53	1.49
Carcass weight (kg)	367	374	377	374
Marbling score	484	470	489	493
Longissimus dorsi area at 12th rib (cm ²)	97.3	99.5	95.6	97.2
Fat (cm)	0.85	0.89	0.99	0.92
Yield grade	1.9	1.9	2.3	2.1

was only 6.5% and carcass composition, longissimus dorsi chemical composition, and meat quality were not affected.

GMP feeding studies in pigs also failed to produce effects on carcass composition and quality. [Hyun et al. \(2004\)](#) compared Roundup Ready corn (NK603) against nontransgenic corn and two commercial corn lines in two separate studies, and the results are summarized in [Table 5.9](#). [Cromwell et al. \(2002\)](#) found that there were no significant differences in the sensory qualities, cooking loss, or sheer forces in pork when pigs were fed glyphosate-tolerant soybean meal or its isogenic non-GM counterpart ([Table 5.10](#)). [Reuter et al. \(2002\)](#) also saw no effects on meat composition and quality when they fed *Bt* maize to pigs.

Table 5.8: Influence of RR-maize (GA21 or NK603) in rations of finishing steers on parameters of meat quality and muscle composition (exp. 1: 75%; exp. 2: 73%; exp. 3: 79.5% maize in finishing diet; 92, 94, and 144 days feeding in exp. 1, 2, and 3; [Erickson et al., 2003](#)).

Parameters	Commercial Maize 1	Commercial Maize 2	Isogenic Control Maize	Transgenic Maize (Exp. 1: GA21; Exp. 2 and 3: NK603)
Final BW (kg)				
Exp. 1	561	575	571	562
Exp. 2	565	570	564	566
Exp. 3	585	575	590	577
Carcass weight (kg)				
Exp. 1	347	356	354	348
Exp. 2	346	348	345	346
Exp. 3	349	364	371	364
Marbling score				
Exp. 1	492	517	485	517
Exp. 2	545	555	559	549
Exp. 3	533	544	539	541
Muscle composition ^a				
<i>Moisture (%)</i>				
Exp. 1	72.3	72.1	73.5	72.3
Exp. 2	71.8	71.7	71.4	71.7
Exp. 3	75.6	75.1	75.2	76.2
<i>Protein (%)</i>				
Exp. 1	22.3	22.0	22.1	22.0
Exp. 2	23.8	23.9	24.0	23.7
Exp. 3	20.5	20.0	20.2	19.6
<i>Fat (%)</i>				
Exp. 1	3.9	4.0	3.5	4.1
Exp. 2	4.0	4.1	4.6	4.3
Exp. 3	3.4	3.9	3.8	3.5

^aExp. 1 and 2: Longissimus dorsi; exp. 3: brachiocephalicus muscle.

[Cromwell et al. \(2005\)](#) later compared diets with high amounts of glufosinolate herbicide-tolerant (LibertyLink) rice against conventional rice diets in growing finishing pigs (72.8, 80.0, and 85.8% in growing, early-finishing, and late-finishing phase, respectively) and saw no effects on carcass quality.

[Taylor et al. \(2004\)](#) compared Roundup Ready (RT73) canola meal, a nontransgenic control, and six commercial canola meals in broilers and observed no effects on the slaughtering results or body composition ([Table 5.11](#)). Most parameters of GM-fed animals were in the same range as animals fed with commercial varieties.

[Stadnik et al. \(2011\)](#) investigated the influence of GM maize (MON810) and/or GM soybean meal (Roundup Ready; [Swiatkiewicz et al., 2010a,b, 2011](#), for experimental design) on the

Table 5.9: Influence of RR-maize (NK603) in grower I and II (68.1 and 74.2%) and finisher I and II (78.1 and 81.8% maize of diet) diets of two studies with pigs on parameters of meat quality and composition (final weight: 116 and 119 kg in exp. 1 and 2; [Hyun et al., 2004](#)).

Parameters	Commercial Maize 1	Commercial Maize 2	Isogenic Control Maize	Transgenic Maize (NK603)
Average weight gain (g/day)				
Exp. 1	910	909	912	912
Exp. 2	985	945	942	954
Longissimus dorsi area (cm ²)				
Exp. 1	58.6	55.3	56.6	56.6
Exp. 2	43.4	41.9	42.9	42.2
Muscle composition (%)				
<i>Moisture</i>				
Exp. 1.	72.4	72.3	72.7	72.5
Exp. 2	73.4	73.2	73.3	73.2
<i>Protein</i>				
Exp. 1	23.5	23.7	23.8	23.5
Exp. 2	23.1	22.9	23.4	23.4
<i>Fat</i>				
Exp. 1	3.06	3.08	2.20	2.99
Exp. 2	2.46	2.83	2.78	2.77

Table 5.10: Effect of glyphosate-tolerant (RR) soybean meal on parameters of pork quality ([Cromwell et al., 2002](#)).

Sensory Parameters ^a	Control	GM
Juiciness	5.52	5.58
Tenderness	5.91	6.10
Off flavor	7.08	7.18
Flavor intensity	5.74	5.95
Connective tissue	3.95	3.58
Cooking loss (%)	32.0	30.3

^aPanel scale 1–8, with 8 being the best.

physical–chemical properties of breast and thigh muscles in broilers ([Table 5.12](#)). Apart from the a* color of the breast muscles, no other parameters were affected by the incorporation of transgenic corn or soybean meal into the broiler's diets.

Further studies using other GM plants (e.g., soybean, maize, wheat, and canola) also failed to show differences in the body composition or quality parameters of broiler meat ([Aulrich et al., 2001](#); [FASS, 2016](#); [Kan and Hartnell, 2004a,b](#)).

[Brown et al. \(2003\)](#) reported that GMP feed did not influence the body composition and meat quality of rainbow trout.

Table 5.11: Selected parameters of carcass yield and composition of broilers fed glyphosate-tolerant canola meal (RT173), nontransgenic control, and six commercial canola meals (starter: 25; grower/finisher: 20% of diet; 100 broilers per treatment; mean values of combined males and females; Taylor et al., 2004).

Parameters	Control	Range of Six Commercial Varieties	Glyphosate-Tolerant RT73
Chill weight (kg/bird)	1.55	1.54–1.60	1.60
Breast meat (% of chill weight)	25.17	25.04–25.63	24.93
Thigh meat (% of chill weight)	15.55	16.44–16.89	16.66
Breast meat analysis			
Moisture (%)	75.10	74.96–75.37	75.25
Protein (%)	23.71	23.65–23.97	23.74
Fat (%)	0.86	0.76–0.92	0.82

Table 5.12: Effects of GM corn (MON810) and RR soybeans (MON-40-30-2) on the physicochemical properties of breast muscle in male broilers ($n = 20$; Stadnik et al., 2011).

Treatments/ Parameters	Control	+GM Soybean Meal	+GM Maize	+GM Soybean Meal and +GM Maize
pH	5.65	5.79	5.76	5.77
Water holding capacity (%)	49.9	55.2	49.9	52.5
Oxidation–reduction potential (mV)	363.1	350.3	333.9	355.7
Color parameters				
L*	56.2	53.6	56.3	56.4
a*	0.79	0.34	1.40	0.56
B*	9.6	9.7	9.7	9.9
TBARS (mg/kg) ^a	1.12	1.04	0.72	0.74

^aThiobarbituric acid reactive substances (TBARS) as a parameter for lipid oxidation.

The large number of feeding studies with first-generation GMPs in food-producing animals and the consistency of the results in 2000–12 (Tables 5.4–5.12, summarized in Table 5.13) has meant that only a few feeding studies using these GMPs have been carried out during the last few years (Czerwinski et al., 2015a,b). These studies confirmed the earlier findings.

Stacked GM events are produced by combining the individual events through conventional breeding and they also require a risk assessment for their use in feeds and foods. Han et al. (2016), Qi et al. (2012), and Zhu et al. (2013) investigated the potential subchronic food safety of stacked traits and concluded that they are as safe as conventional non-GM plants. Kramer et al. (2016) analyzed data from 15-year worth of studies on stacked GM events

Table 5.13: Summary of published data in peer reviewed journals to compare feeds from GM plants of the first generation (with input traits) with their isogenic counterparts in food-producing animals (Flachowsky, 2013, 2015).

Animals (Species/Categories)	Number of Experiments	Nutritional and Safety Assessment
<i>Ruminants</i>		No unintended effects on the composition and contamination of GMPs compared with isogenic counterparts (except lower mycotoxin concentrations in GMP with <i>Bt</i>)
Dairy cows	23	
Beef cattle	14	
Others	10	
<i>Pigs</i>	21	No biologically relevant effects on feed intake, digestibility, or animal health, and no unintended effects on the performance and fertility of animals or on the composition and quality of foods of animal origin. Recombinant DNA and newly expressed proteins in GMP do not show other chemical/physical properties as native substances
<i>Poultry</i>		
Laying hens	10	
Broilers	28	
<i>Others</i>		
(Fish, rabbits, etc.)	8	

in maize and came to the conclusion that these events do not alter the composition, protein expression, or inert stability.

In summary, feeds from first-generation GMPs can be considered as substantial equivalents to their isogenic counterparts (Flachowsky, 2013; Mejia et al., 2010; van Eenennaam and Young, 2014).

4 Feeding Studies With GM Plants of the 2nd Generation

GMPs of 2nd generation are able to improve animal nutrition by providing nutrients without the need for animal feed supplementation. Biofortified crops may be characterized by higher protein content or changed amino acids patterns (Lucas et al., 2007; Sevenier et al., 2002; Ufaz and Galili, 2008), higher fat or modified fatty-acid content (Cahoon et al., 2007; Napier, 2007), altered vitamin precursors and/or vitamin content (Beyer et al., 2002; DellaPenna, 2007; Potrykus, 2001; van Eenennaam et al., 2004; Ye et al., 2000), or altered mineral content (Broadley et al., 2006; Goto et al., 1999; Lucca et al., 2001; Welch and Graham, 2004; White and Broadley, 2005). They can also help to alleviate malnutrition by providing important micronutrients (Bouis, 2016; Bouis et al., 2003; Maruyama et al., 2011; Mayer et al., 2008; Nestel et al., 2006; Parisi et al., 2016; Pfeiffer and McClafferty, 2007; Ricroch and Henard-Damave, 2015; Tillie et al., 2013; Welch and Graham, 2004).

Some nutrients may influence the composition and quality of animal-origin foods (e.g., fatty acids) while others may be deposited in various organs (e.g., vitamin A and Cu in the liver) or animal products (e.g., iodine in milk and eggs and vitamin E in eggs; Table 5.1.). Table 5.14 shows the nutrients (bold print) that are especially important for human and animal nutrition.

Table 5.14: Grouping and essential nutrients for humans and animals.

Groups of Nutrients	Essential Nutrients
Amino acids	Histidine, isoleucine, leucine, lysine , methionine , phenylalanine, threonine , tryptophan , and valine (semi-essential: arginine and cysteine)
Fatty acids	Linoleic acid and linolenic acid
Major elements	Ca , Mg , P , Na, K, S, and Cl
Trace elements	Fe , Zn , Cu, Mn, I , Se , and Co (cobalamin; vit B ₁₂)
Ultratrace elements ^a	F, B, Mo, Ni, Cr, V, Si, As, Cd, Pb, Li, Sn
Vitamins (fat soluble and water soluble)	A (precursor of β -carotene), D, E , and K B ₁ (thiamin), B ₂ (riboflavin), B ₆ (pyridoxin), B₁₂ (cobalamin), pantothenic acid, niacin, folate , biotin, and C (ascorbic acid)

Bold letters: high importance for human nutrition.

^aEssentiality of some elements is unclear (McDowell, 2003); occasionally beneficial elements (Suttle, 2010).

There are large differences in the assessment of plants to be used for the biofortification of human and animal diets. For animal nutrition, many feed additives are available to complete animal diets. In addition to essential nutrients, there is also a large group of feed additives that are nonessential, but which may help in feed preparation (technological additives), feed intake (sensory additives), and/or feed conversion (zootechnical additives). Reasons to add such nonessential additives to animal feeds are summarized in Fig. 5.2.

Nonessential feed additives can be classified into three major groups:

1. Technological additives: these are substances that improve the conservation of feeds, such as silage additives, antioxidants, emulsifiers, stabilizers, gelling agents, and acid regulators.

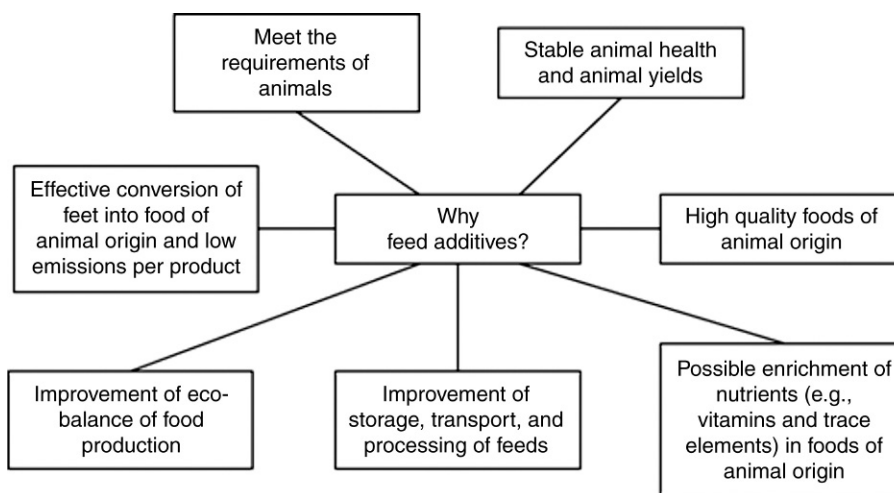


Figure 5.2: Why Feed Additives in Animal Nutrition?

2. Sensory additives: include colorings, flavorings, and appetizers.
3. Zootechnical additives: these are substances that improve the nutrient digestibility and/or nutrient conversion (i.e., enzyme and microorganisms), and substances that influence the digestive-tract microbiota in a wished direction (i.e., growth promoters).

The Panel for Feed Additives and Products (FEEDAP-Panel) of the European Food Safety Authority (EFSA) is responsible for assessing the safety of feed additives for humans, animals, and the environment. The FEEDAP-Panel distinguishes nonessential feed additives as:

- Coccidiostats and histomonostats
- Coloring agents
- Enzymes
- Feed flavorings
- Microorganisms (probiotics)
- Mycotoxin detoxifying agents
- Organic acids
- Other zootechnical additives
- Silage additives
- Technological additives

The effects of various feed additives in animals, animal-origin foods, and on the environment are described in detail in various reviews, textbooks, publications, and in EFSA opinions in the EFSA Journal ([Bunglavan et al., 2014](#); [Flachowsky and Meyer, 2015](#); [Flachowsky et al., 2013](#); [Pape, 2006](#); [Salem, 2011, 2012](#)).

[Table 5.15](#) summarizes the pros and cons of biofortification in various plants. In summary, biofortification is not so important from the perspective of animal nutrition, but it may be helpful in meeting human nutritional requirements. It could also be considered in the fight against micronutrient malnutrition in humans ([Zimmermann et al., 2004](#)).

Apart from essential nutrients ([Table 5.14](#)), the content of nonessential ingredients (i.e., enzymes) may also be increased through biofortification ([Gao et al., 2012](#); [Nyannor](#)

Table 5.15: Pros and contras of substantial changes in plant composition (plants of the second generation; plants with output traits or biofortified plants).

Pros	Contras
More advantageous for human nutrition (meet fatty acid, mineral, and vitamins requirements) than animal nutrition (except for smallholders or farmers who reside far from transport links)	Plant breeding takes a long time (longer than developing a food/feed additive)
Lower content of undesirable substances	Many feed additives are available for animal nutrition
Improved properties of foods/feeds	High amounts of foods/feeds may be necessary to meet requirements

et al., 2007; Zhang et al., 2000). The concentrations of nondesirable substances (i.e., phytate, glucosinolates, or mycotoxins) (Diaz-Gomez et al., 2015; Duvick, 2001; Munkvold et al., 1999; Vageeshbabu and Chopra, 1997; Zhu et al., 2016) may be reduced and this may influence the health and performance of the animal and the composition and quality of animal-origin foods.

Special attention has been made to modify the fatty-acid pattern in GM plants (e.g., oleic acid instead of linoleic acids in oilseeds; Brown et al., 1999) or to produce “unusual” fatty acids in transgenic plants (Cahoon et al., 2007). Very long chain polyunsaturated fatty acids are normally found in fish oils and marine microorganisms.

4.1 Increased Content of Amino Acids

Some feeds are characteristically deficient in essential amino acids, such as lysine, methionine, threonine, and/or tryptophan. The first limiting amino acid in maize is lysine; therefore, lysine-rich feeds and/or lysine supplementation are required for optimal performance and carcass characteristics of birds and pigs (NRC, 1994, 1998; GfE, 1999, 2008). One of the objectives of traditional plant breeding was to increase the lysine content in maize. Information regarding a gene that changed protein fractions and increased the lysine content in maize endosperm was already known in the 1960s (Mertz et al., 1964; Nelson et al., 1965; Wolf et al., 1967). High-lysine mutants were later named opaque-2 and floury-2 (Christianson et al., 1974; Sodek and Wilson, 1971); however, cultivation with these mutants was stopped due to their sensibility to some diseases, problems during harvest, and reduced yields (Hoffmann et al., 1985).

Modern biotechnology has also provided alternatives for dietary amino acid supplementation. For example, the development of lysine maize was a step in the right direction for improving broiler feeding. Lucas et al. (2007) used high portions of lysine maize (LY038 and LY038×MON810) in broiler diets (59.2 and 66.1% in starter and grower–finisher diets, respectively), and compared these strains with nonsupplemented and lysine-supplemented control maize, and five commercial maize varieties (nonsupplemented and lysine supplemented). Carcass characteristics and body composition were not significantly different between lysine maize and lysine-supplemented control maize; however, the nonsupplemented control chicks had lower body weights (Table 5.16). This research suggests that the

Table 5.16: Carcass characteristics of broilers fed with control maize, lysine-supplemented control maize, and lysine maize (Lucas et al., 2007).

Parameters	Control	Control + Lysine	LY038	LY038 × MON810
Chilled weight (g)	1,156	1,601	1,591	1,561
Breast weight (g)	222	347	349	333
Breast meat composition (%)				
Moisture	77.6	77.6	77.3	77.3
Protein	19.2	19.8	20.0	19.6
Fat	2.17	1.58	2.35	1.85

bioefficacy of the incremental lysine in LY038 and LY038 $\times$ MON810 was not different from that of lysine in conventional maize diets supplemented with L-lysine HCl.

4.2 Expression and Uses of Stearidonic Acid in Soybeans (C18:4 $n-3$; SDA)

Altered composition of GMPs and derived feeds may influence the composition of animal-origin foods, as has been demonstrated in soybeans with a modified fatty-acid pattern. The introduction of two genes modified the expression of $\Delta 6$ and $\Delta 15$ -desaturases and the biosynthesis of a highly unsaturated fatty acid with four double bonds, which naturally occurs in *Echium* oil (Surette et al., 2004). This SDA (C18:4 $n-3$) may be transferred into the body fat of nonruminants or used as a precursor for longer chain fatty acids (e.g., C20:5 $n-3$, eicosapentaenoic acid; EPA; and C22:6 $n-3$, docosahexaenoic acid) in nonruminants (Tables 5.17–5.19), fish (Cleveland et al., 2012), ruminants, and humans (Banz et al., 2012; Harris et al., 2007, 2008; James et al., 2003). The conversion efficacy of SDA to EPA varied between 3:1 and 6:1 (James et al., 2003; Whelan et al., 2012).

Stearidonic soybean oil contains 20%–30% SDA (Rymer et al., 2011). Rymer et al. (2011) added 45g of SDA-oil (containing 24% SDA/kg) to broiler grower diets and 50 g to finisher diets and compared this with conventional soybean oil and fish oil. The authors did not observe any significant influence of SDA-oil on feed intake, weight gain, or feed conversion rate, but they did find higher concentrations of SDA and C₂₀ and C₂₂ polyunsaturated fatty acids in various body fats (Table 5.17). Table 5.18 shows the influence of added oils on the body pool size of $n-3$ fatty acids in broilers.

Finally, Rymer et al. (2011) investigated the influence of soybean oil, SDA oil, and fish oil on the sensory attributes of breast and leg meat. Aroma, taste, and aftertaste of the freshly cooked breast meat were not significantly influenced by the oil source. Fishy aromas, tastes, and aftertastes were associated with higher concentrations of $n-3$ fatty acids in the meat (Table 5.18). This was most noticeable in leg meat after fish oil supplementation, but it was also present in the reheated leg meat of the SDA-group. More studies are necessary to overcome this weakness.

Similar results regarding the transfer of SDA in milk were described by Bernal-Santos et al. (2010) in lactating cows after duodenal infusion of SDA soybean oil; by Kitessa and Young (2011) after feeding of rumen protected SDA oil to dairy cows (Table 5.19); by Mejia et al. (2010) in laying hens; and by Forster et al. (2011) in pacific white shrimp. Gibbs et al. (2010) considered introducing SDA oils in animal feed to increase the intake of long-chain $n-3$ PUFA in men.

However, some polyunsaturated fatty acids have no upper limit in human nutrition and care should be taken when supplementing these oils in animal feeds (Hammond et al., 2008; Harris et al., 2007; Miles et al., 2004; Schubert et al., 2007). Therefore, animal body

Table 5.17: Concentrations of $n-3$ fatty acids (mg/100 g fresh tissue) in the body samples of broilers (unsupplemented control or supplemented with fish oil or SDA oil; Rymer et al., 2011).

Samples	Control	+45 (Grower) and 50 g Fish Oil (Finisher Diet)/kg Feed	+45 (Grower) and 50 g SDA-Oil (Finisher Diet)/kg Feed
Fat content (g/kg)			
Breast meat	39.9	29.9	39.4
Leg meat	68.0	67.4	70.7
Skin	481.0	422.0	465.0
Breast meat			
C18:4 $n-3$	3	13	231
C20:5 $n-3$	12	49	28
C22:6 $n-3$	7	107	14
Leg meat			
C18:4 $n-3$	10	36	442
C20:5 $n-3$	5	141	53
C22:6 $n-3$	8	185	21
Skin			
C18:4 $n-3$	111	286	3,673
C20:5 $n-3$	31	1,058	317
C22:6 $n-3$	21	921	78

Table 5.18: Calculation on the influence of added oils on the intake and the pool size of $n-3$ fatty acids in edible tissues (breast and leg meat with skin) in broilers (Rymer et al., 2011).

Fatty Acids	Control	Supplemented Fish Oil	Supplemented SDA Oil
Intake of $n-3$ fatty acids (mg/broiler)			
C18:3	17,383	6,415	19,717
C18:4	0	3,133	51,219
C20:5	0	13,724	0
C22:5	3	4,919	7
C22:6	6	13,611	0
Pool size of fatty acids in edible tissues (mg/broiler)			
C18:3	3,840	1,662	4,884
C18:4	227	690	9,645
C20:5	155	2,627	975
C22:5	234	1,777	1,126
C22:6	131	3,110	331

composition may also be considered as an endpoint in animal feeding studies to measure the transfer of second-generation GMP ingredients into tissues, milk, or eggs (Table 5.2.). Animal body samples or products (i.e., milk and eggs) should be considered and analyzed adequately. Furthermore, efficiency studies with second-generation GMPs may be used as, or combined with, studies to measure the digestibility/bioavailability of the newly expressed substances.

Table 5.19: Fatty-acid concentrations (mg/L) of some fatty acids in whole milk on the initial and the final day (10th day) of supplementation with ruminally protected SDA-oil (offered about 40 g C18:4*n*-3 per cow and day; SDA; intake about 30 g; *n* = 5; [Kitesa and Young, 2011](#)).

Fatty Acids	Initial Value (Day 1)	Final Values (Day 10)
C18:1 <i>n</i> -9; oleic acid	8,880	18,582
C18:2 <i>n</i> -6; linoleic acid	600	746
C18:3 <i>n</i> -3; linolenic acid	463	877
C18:4 <i>n</i> -3; stearidonic acid	18	144
C20:5 <i>n</i> -5; EPA	13	76
C22:5 <i>n</i> -3; DPA	45	65
Total <i>n</i> -3	559	1,162
Total <i>n</i> -6	622	886

4.3 Conjugated Linoleic Acids

Apart from the transfer of fatty acids into animal products, some fatty acids (e.g., conjugated linoleic acids, CLAs) may influence fatty depositions in animal and human bodies ([Akahoshi et al., 2005](#); [Ostrowska et al., 2005](#)) or trigger specific health effects ([Lunn and Theobald, 2006](#)).

Such influences have been reported for GM rapeseed ([Hornung et al., 2002](#); [Iwabuchi et al., 2003](#); [Kohno-Murase et al., 1994](#)) containing the CLA isomer *cis*-9, *trans*-11, *cis*-13-octadecatrienoic acid (punicic acid). The oil was added to the diets of mice (0.25% puniic acid) and it affected a significant decrease in white adipose tissue from 0.94 (control) to 0.61 g/100 g of body weight ([Koba et al., 2007](#)). Punicic acid was also found in the fatty acid profiles of liver triglycerides and liver phospholipids of mice fed with the GM rapeseed oil. These result show that it would be possible to produce CLA via plants.

4.4 Increasing Concentrations of Micronutrients (the Vitamin A Precursor β -Carotene)

Deficiencies in trace elements (e.g., iodine, iron, and zinc) and vitamins (e.g., A, E, B₁₂, and further B-vitamins) in humans and animals are serious problems in many countries ([Fitzpatrick et al., 2012](#); [Jones and de Brauw, 2015](#)). Green biotechnology (i.e., biofortification) may help to overcome these problems ([DellaPenna, 1999](#); [Trijatmiko et al., 2016](#); [Zimmermann et al., 2004](#)).

Interesting results have been reported with “Golden Rice,” which is rich in the vitamin A precursor β -carotene ([Dawe et al., 2002](#); [Potrykus, 2001](#); [Ye et al., 2000](#)). Table 5.20 summarizes the results from a study where the bioconversion of β -carotene into vitamin A was assessed in Mongolian gerbils. After an initial depletion period, gerbils were fed with different diets and the results show that the hepatic retinol concentration in gerbils fed with

Table 5.20: Experimental design to assess the conversion of β -carotene from maize into vitamin A in Mongolian gerbils (60% maize in diets; $n = 10$; depletion phase: 4 weeks; feeding: 8 weeks; Howe and Tanumihardjo, 2006).

	Nonsupplemented Control (Maize Low in Carotene)	Maize Rich in Carotene	Control + β -Carotene	Control + Vitamin A
β -Carotene (nmol/g)	0	8.8	8.8	4.4
Theoretical retinol intake (nmol/day)	0	106	106	106
Retinol in serum (μ mol/L)	1.23 ± 0.20	1.25 ± 0.22	1.23 ± 0.20	1.22 ± 0.16
Retinol in liver (μ mol/g)	$0.10^a \pm 0.04$	$0.25^b \pm 0.15$	$0.25^b \pm 0.08$	$0.56^c \pm 0.15$

^{a,b,c} Means with different letters differ ($P < 0.05$).

carotene-rich maize was similar to those animals fed with maize lacking in β -carotene and those supplemented with adequate amounts of β -carotene.

“Golden Rice” can contain between 1.6 (Ye et al., 2000) and 37 mg of β -carotene/kg of dry rice (Paine et al., 2005). The first studies to determine the vitamin A value of β -carotene were performed using deuterium-labelled rice in five human subjects (Tang et al., 2009) and not in laboratory or target animals. Tang et al. (2012) compared β -carotene in “Golden Rice” with pure β -carotene and β -carotene in green spinach, as a method for providing vitamin A to children ($n = 68$; 6–8 years). The conversion of β -carotene to vitamin A was as effective in “Golden Rice” as pure β -carotene (2:1 and 2.3:1 by weight, respectively); however, spinach exceeded both of these (7.5:1) (Tanumihardjo, 2002). Albaugh (2016) concluded that the “Golden Rice” was an effective and safe method for providing vitamin A.

Although biotechnologies may contribute to biofortification of feeds and food, they could also increase the storage capacity and shelf life, especially for fruits (van de Weg et al., 2016).

4.5 Lower Content of Undesirable Ingredients

Supplementation of animal diets with inorganic P and supplementation of feed with the microbiologically synthesized enzyme phytase are methods that allow dietary P requirements to be met (Rodehutsord et al., 1996; Wodzinski and Ullah, 1996). However, there are also opportunities in plant and animal breeding to improve P availability. These include:

- Reducing phytate synthesis in plants to create low phytate hybrids of maize, barley, rice, or soybeans (Spencer et al., 2000a,b).
- Expressing phytase in plants and a higher bioavailability of phytate P (Gao et al., 2012).

Table 5.21: Conventional and low-phytate maize (78.5 % of the mixture) in the feed of fattening pigs.

Parameters	Control		Low-Phytate Maize	
	(0.3 g of Available P/kg)		(1.7 g of Available P/kg)	
Inorganic P supplement	—	+	—	+
P content (g/kg)				
29–73 kg live weight	3.4	5.4 ^a	3.4	5.4 ^a
73–112 kg live weight	3.2	4.7	3.2	4.7 ^b
Feed intake (kg/day)	2.23 ^A	2.50 ^B	2.53 ^B	2.51 ^B
Live weight gain (g/day)	730	870 ^B	900 ^B	880 ^B
Feed per gain (kg/kg)	3.05 ^A	2.87 ^B	2.81 ^B	2.85 ^B
P excreted (g/kg)	4.6 ^A	8.9 ^C	3.8 ^B	8.8 ^C
Strength (fourth metacarpal bone, kg)	79.3 ^A	138.5 ^{B,C}	132.2 ^B	153.9 ^C
Ash content (% in the fourth metacarpal bone)	53.5 ^A	60.1 ^{BC}	59.3 ^B	61.2 ^C

^{A,B,C} Different letters in one line indicate significant differences ($P < 0.05$).

^a2 gP/kg.

^b1.5 gP/kg.

Source: From Spencer, J.D., Allee, G.L., Sauber, T.E., 2000a. Phosphorus bioavailability and digestibility of normal and genetically modified low-phytate corn for pigs. *J. Anim. Sci.* 78, 675–681; Spencer, J.D., Allee, G.L., Sauber, T.E., 2000b. Growing-finishing performance and carcass characteristics of pigs fed normal or genetically modified low-phytate corn. *J. Anim. Sci.* 78, 1529–1536.

- Expressing phytase in the digestive juice of nonruminants.
- Reducing undesirable plant ingredients and mycotoxins.

In a porcine study, low phytate maize showed the same results as traditional maize supplemented with 2 or 1.5 g inorganic P/kg of feed, but a significantly lower P excretion (Table 5.21; Spencer et al., 2000a,b). No influence on the composition or quality of pork was observed. More studies relating to transgenic crop feeding in livestock can be found in FASS (2016).

5 Conclusions

A reevaluation of studies that used GMPs as feeds in animal nutrition showed that they have no negative effects on the composition or quality of animal-origin foods (Panchin and Tuzhikov, 2016). Twenty years after the first commercial cultivation of GM crops (1996–2015), some authors have analyzed the socioeconomic, environmental, and health effects of these crops and established that they are of benefit to farmers, consumers, and innovators (Brookes and Barfoot, 2016; JRC, 2016; Klümper and Qaim, 2014; Qaim, 2016; Smyth, 2014; Smyth et al., 2015; Tabashnik et al., 2013; The Royal Society, 2016; van Eenennaam, 2013; Watson and Preedy, 2015). However, further developments in green biotechnology are necessary to guarantee advantages for all partners and for ensuring food security and food safety (Bartsch et al., 2015; Bearth and Siegrist, 2016; Conko et al., 2016; NASEM, 2016).

All methods of plant breeding that contribute to the more resource-efficient production of high and stable yields of available biomass should be used to produce feed for food security and safety. NASEM (2016) recommended that new varieties, whether genetically engineered or conventionally bred, should be subjected to safety testing if they have novel characteristics (intended or unintended) with potential hazards.

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Genetically Modified Microorganisms: Harmful or Helpful?

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1 Introduction

Microorganisms have been associated with human activity for thousands of years. One of the oldest known food preservation methods is fermentation, a process aimed at conserving foods and producing a wide range of food products. Although the exact time at which people began to produce fermented products cannot be established, it is assumed that this activity started with animal domestication (Demain, 2010).

Despite the extensive use of fermentation in everyday life, understanding of the identification, classification, and metabolic activities of the microorganisms involved in the process have only been developed during the last 200 years. In 1680 Anton van Leeuwenhoek was the first to observe yeast cells through a microscope. He did not make the connection between these microbes and fermentation, as most chemists believed that the process was due to a catalytic reaction. It was not until 1857 that Louis Pasteur discovered that fermentation is caused by living organisms and in 1897, Eduard Buchner succeeded in extracting the responsible enzyme from yeast cells. Scientists have since turned their attention to microorganisms involved in fermentation and more specifically their metabolism. It has been shown that the fermentation processes for obtaining food products are very complex and usually involve multiple types of microorganisms, such as yeast, lactic acid bacteria (LAB), and fungus (Chojnacka, 2010; Demain, 2010).

The industrial revolution, the demographic boom, and the globalization of the food and agricultural market has led to a growing need for processed food, food products, and additives with high-nutritional value and minimal-health risks. The emergence and development of genetic engineering during the past few decades has gone some way to solving some of these problems. Information and techniques aimed at understanding and modifying the genetic material of the organisms has produced microbial strains with improved or novel properties that have high potential for use in industry (i.e., food, chemical, and pharmaceutical), biotechnology, and medicine. Genetic modification of microbial strains usually acts to increase

the production of desired compounds or limit the release of undesirable metabolic products. Thus the possibility of introducing genetically modified organisms (GMOs) and genetically modified microorganisms (GMMs) into the food industry represents an interesting solution for meeting the market demands. Conversely, GMMs could revolutionize the food industry by generating products of superior quality with more pronounced tastes and smells and pleasant textures. However, despite the huge number of research studies concerning the amelioration of microorganisms through genetic engineering techniques, at present only a few GMM strains are commercially used or pending approval. This is mainly due to the reluctance of the public regarding this subject and to legal restraints. Therefore attempts are being made to use alternative techniques for the genetic and metabolic amelioration of food microorganisms.

This chapter deals with the incidence and use of LAB and yeasts in foods, some of the major improvements in their metabolic characteristics using genetic engineering and other manipulations, and aims to analyze the extent of the harmful issues related to this subject.

2 Incidence of Microorganisms in Foods

2.1 Lactic Acid Bacteria

People consume food products obtained from microbial fermentation (i.e., bread, dairy products, wine, beer, and pickles) on a daily basis. Food fermentation can be classified based on the pH and whether the end product is alcoholic, lactic, or alkaline. Alcoholic fermentation takes place in yeast strains and ethanol is the main product of sugar metabolism; alkaline fermentation involves ammonia production by amino acid deamination and is usually carried out by different species of *Bacillus* and *Staphylococcus*; and lactic fermentation results in the production of lactic acid and occurs in strains of LAB (Demain, 2010).

LAB strains have been used by humans for food production and preservation for years and have recently been employed for their therapeutic role. LAB are Gram-positive, nonspore-forming bacteria that are naturally present in plants, dairy products, and the gastrointestinal tract of humans and animals (Florou-Paneri et al., 2013).

LAB strains are currently used as starter cultures in the manufacture of dairy products (i.e., yogurt, cheese, kefir, and buttermilk), meat (i.e., ham and sausage), bakery products, alcoholic beverages (i.e., beer and wine), and vegetable processing (i.e., pickles, olives, and cocoa beans) (Table 6.1) (Boyacioglu et al., 2010; Darsonval et al., 2016; Florou-Paneri et al., 2013; Macedo et al., 2012; Scheirlinck et al., 2007; Tamime and Robinson, 2007). During fermentation some strains of LAB are able to release metabolites into the environment, which have beneficial effects on flavor, shelf life, texture, and the nutritional value of the food product. LAB strains are also able to release antimicrobial compounds and are capable of inhibiting the growth of other microbial contaminants (Kongo, 2013). A large number of research papers have demonstrated that some members of the LAB group (especially

Table 6.1: LAB species incidence and activity in foods.

LAB Species	Food Products	Important Compounds
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> <i>Streptococcus thermophilus</i> <i>Lactococcus lactis</i> biovar <i>diacetylactis</i> <i>L. lactis</i> subsp. <i>cremoris</i> <i>Oenococcus oeni</i>	Yogurt	Lactate, exopolysaccharides, and aroma compounds (acetaldehyde, acetoin, and diacetyl)
<i>Lactobacillus</i> , <i>Leuconostoc</i> , <i>Pediococcus</i> , <i>Weissella</i> species	Wine Bakery	Lactic acid, diacetyl, volatile phenols, and sulfur
<i>L. curvatus</i> , <i>L. sakei</i> , <i>L. acidophilus</i> , <i>Lactobacillus alimentarius</i> , <i>Lactobacillus brevis</i> , <i>L. casei</i> , <i>L. fermentum</i> , <i>L. plantarum</i> , <i>Lactobacillus pentosus</i> <i>L. lactis</i> <i>P. acidilactici</i> , <i>Pediococcus pentosaceus</i> <i>L. plantarum</i> <i>Leuconostoc</i> spp. <i>Pediococcus</i> , <i>Lactococcus</i> species	Meat	Esters, alcohols, aldehydes, and organic acids
<i>Lactobacillus rhamnosus</i> , <i>L. casei</i> , <i>L. acidophilus</i> , <i>Lactobacillus johnsonii</i> , <i>L. plantarum</i> , <i>L. fermentum</i> , <i>Lactobacillus reuteri</i>	Fermented vegetables Probiotics	Lactic acid Organic acids, bacteriocins, short-chain fatty acids, and adhesins

LAB, Lactic acid bacteria.

lactobacilli) can exhibit prophylactic and therapeutic properties in humans and animals, some of which are now included in probiotic products.

2.2 Yeasts

Yeasts represent the more extensively used class of microorganisms, and they have been associated with the evolution of human society. Archaeological studies revealed that wine production began almost 7000 years ago in Mesopotamia, while 3000–4000-year-old artefacts from beer production and grain fermentation have been found in Summer, Mesopotamia, Egypt, and Babylon. Bread making seems to date back to 6000 years, while the use of dough in baking seems to have been established in approximately 500 BC (Mortimer, 2000). The term yeast originates from medieval times and is related to the fermentation of these microorganisms, reflecting a long-term preoccupation with using their specific metabolic abilities for the benefit of mankind. The first complex study on yeast classification and characterization was published in 1952, comprised 164 species, while the most recent study describes more than 1500 species and 149 genera (Kurtzman et al., 2011).

The importance of yeasts in foods and beverages refers not only to their use in baking or the production of beer and wine, but extends to obtaining processed foods, food ingredients,

additives, probiotic compounds, and biocontrol agents (Table 6.2). In contrast, the spoilage of raw or processed foods by various yeast species is a constant concern (Boekhout and Phaff, 2003; Breuer and Harms, 2006; Buerth et al., 2016; Fleet, 2006; Gonçalves et al., 2014; Tamang and Fleet, 2009; Tataridis et al., 2013).

Table 6.2: Yeast species used in food industry and for production of diet supplements.

Genus	Species	Incidences	Activities
<i>Brettanomyces</i> (teleomorph <i>Dekkera</i>) <i>Candida</i> (<i>Torulopsis</i>)	<i>Brettanomyces bruxellensis</i> <i>Dysthaeta anomala</i> <i>Candida parapsilosis</i> <i>C. rugosa</i> <i>C. boidinii</i> <i>C. famata</i> <i>C. krusei</i>	Cider, wine, soft drinks, beer, and dairy products Soft drinks, dairy, fresh and processed meats, cereal products, sourdough, and breads	Spoilage and production of aromas for beer Spoilage, fermentation of fruit juice and coffee, synthesis of lipase, production of flavors, and a possible probiotic agent
<i>Cryptococcus</i>	<i>C. laurentii</i> <i>C. albidus</i>	Fruits and fresh meat	Biocontrol spoilage
<i>Debaryomyces</i>	<i>Debaryomyces hansenii</i>	Processed meat and cheese	Fermentation of meat products and halotolerant food spoiling
<i>Filobasidium</i>	<i>Filobasidium floriforme</i> <i>F. elegans</i> <i>F. globisorum</i> <i>F. capsuligenum</i>	Plants and wine	Synthesis of extracellular α -amylase and glucoamilase and production of pectinases
<i>Galactomyces</i>	<i>G. aurantii</i> <i>G. geotrichum</i> <i>G. reessii</i>	Plants and dairy	Spoilage, extracellular lipase synthesis, and cheese fermentation
<i>Hanseniaspora</i> (anamorph <i>Kloeckera</i>)	<i>H. uvarum</i> <i>H. occidentalis</i> <i>H. guilliermondii</i>	Wine, orange juice, and cider	Augmented fermentative ability
<i>Kluyveromyces</i>	<i>K. lactis</i> <i>K. marxianus</i> <i>K. thermotolerans</i>	Dairy and bakery products	Lactose fermentation to obtain probiotic compounds, inulinase production, food flavors and additives, and frozen dough breads
<i>Metschnikowia</i>	<i>M. pulcherrima</i> <i>M. fruticola</i>	Grapes, fruits, and wine	Antagonistic activity for biocontrol and fermentation
<i>Pichia</i>	<i>P. anomala</i> <i>P. guilliermondii</i> <i>P. membranefaciens</i> <i>P. jadinii</i>	Fruits, grapes, meat products, and bread	Antagonistic activity used in biocontrol, a possible probiotic agent, and spoilage
<i>Rhodotorula</i>	<i>R. glutinis</i> <i>R. mucilaginosa</i> <i>R. graminis</i> <i>R. minuta</i>	Fruits, dairy, and processed meat	Biocontrol Food flavors
<i>Saccharomyces</i>	<i>S. cerevisiae</i> <i>S. uvarum</i> <i>S. bayanus</i> <i>S. pastorianus</i> <i>S. boulardii</i>	Bread, beer, wine, and dairy products	Baking, beer production, wine, and obtaining probiotic compounds

Table 6.2: Yeast species used in food industry and for production of diet supplements. (cont.)

Genus	Species	Incidences	Activities
<i>Saccharomycopsis</i>	<i>S. fibuligera</i>	Starchy substrates and coffee	Amylolytic activity for fermentation of indigenous flours and fermentation of coffee
<i>Torulaspora</i>	<i>T. delbrueckii</i> <i>T. pretoriensis</i>	Dairy, fruits, and soft drinks	Synthesis of alcohols, esters, terpenes for beer and wine, and frozen dough breads
<i>Trichosporon</i>	<i>T. beigelii</i> <i>T. pullulans</i> <i>T. cutaneum</i> <i>T. dulcitum</i>	Starchy substrates, raw dairy products, meat, and fermented doughs	Spoilage and synthesis of amylase and glucoamilase
<i>Yarrowia</i>	<i>Y. lipolytica</i>	Dairy and processed meats	Food additives, flavours, organic acids, polyalcohols, and surfactants

3 Strategies for the Improvement of Microorganisms in Foods and Beverages

According to the World Health Organization (WHO), GMOs present altered genetic material due to gene transfer from one organism to another using recombinant DNA technology (FAO/WHO, 2001). Recombinant DNA technology produces GMOs and GMMs, with higher molecular diversity and augmented metabolic performances, respectively, which are generally associated with lower production costs. The development of genome and proteome investigation techniques led to a deeper understanding of the microbial genome and metabolic pathways. This has since led to the acquired knowledge being used in industry and biotechnology.

Selecting the appropriate strategy for obtaining useful GMMs is based on the analysis of a desired metabolic or phenotypic trait to establish the modifications required for improving it. The main approaches involve: (1) using vectors and gene transfer methods (Feldmann, 2005; Sybesma et al., 2006) to obtain a new compound by cloning and expressing the coding gene from a foreign organism in an easy-to-grow microorganism, which does not naturally harbor that compound; (2) disrupting the function of undesired genes; (3) overexpressing the desired genes; (4) improving the characteristics of different proteins by site-directed mutagenesis and DNA shuffling; (5) augmenting the efficiency of the process by enhancing the yields of the final products or by-products using mutagenesis or different growth and cultivation parameters (Han, 2004; Kulkarni, 2016).

European Union legislation currently limits the use of GMOs and GMMs in foods to a much larger extent than the United States and others countries. The majority of microbial strains used in the EU food industry market are generally obtained by uncontrolled genetic changes (i.e., random mutagenesis, dominant selection, adaptive evolution, natural conjugation,

and transformation) while microbial strains obtained by controlled genetic techniques (i.e., recombinant DNA) are generally not accepted (Sybesma et al., 2006). The public perception of GMOs also varies considerably around the world. Most people in Europe consider GMOs to be unsafe for human consumption while most people in other countries partially agree with the consumption of GMOs.

To overcome these challenges, classic techniques and manipulations have been optimized and developed to improve the genetic characteristics of LAB and yeast.

Until now there have only been a few genetically modified LAB strains on the market, most of which were obtained by screening tests and mutagenesis.

One important question is how strains of the same species can have different characteristics? This occurs because of reading errors in the genetic material of microbial strains within each round of replication. In addition, horizontal genetic transfer mechanisms can also take place in bacterial cells (i.e., insertion sequence elements, conjugation, transformation, and transduction). All these spontaneous natural mutations may confer novel features to the bacterial strain. The restrictive legislation regarding GMO utilization in the food industry means that starter culture manufacturers isolate new LAB strains with desired characteristics from the environment using screening tests. Adaptive evolution is a technique used to improve microbial strains for the food industry. In this technique the strain is slowly adapted under certain growth conditions to allow growth of only the population with the desired properties. Another method for obtaining genetically improved LAB is by mutagenesis, either physical (UV light) or chemical [N-methyl-N'-nitro-N-nitrosoguanidine, ethyl methyl sulfonate (EMS), or acridine orange]. Exposure of microbial cells to these mutagenic conditions increases the frequency of mutations and is followed by screening tests aimed at selecting colonies with the desired traits. A major disadvantage of these uncontrolled genetic techniques is represented by the fact that microbial strains could acquire unwanted mutations, which can affect other important features (Derkx et al., 2014).

For yeasts, most techniques are based on: (1) sexual hybridization (i.e., direct mating between two haploid cells produced by sporulation, rare mating between cells that have skipped the sporulation step but have been subjected to mating-type switching, or mass mating between cells from multiple strains associated with genome shuffling), or (2) asexual hybridization comprising cytoduction (the transfer of cytoplasmically-inherited characteristics) and protoplast fusion (the formation of hybrid diploids) (Steensels et al., 2014). Other methods include: (1) global transcription machinery engineering, based on obtaining a library of random mutated versions of a global transcription factor or associated factor by simultaneous multiple gene modification (Alper and Stephanopoulos, 2007); (2) adaptive evolution to certain cultivation or growth conditions; and (3) mutagenesis, either chemical (EMS) or physical (UV light).

3.1 Baking Products

Sourdough is used in the manufacture of numerous baked products, such as breads, cakes, and crackers (Aplevicz et al., 2013). Bakery products obtained from sourdough have unique characteristics, such as a stronger acid taste and a more aromatic and crispy crust (Lonner and Preve-Akesson, 1989). LAB strains contribute significantly to these properties by releasing organic acids, protease, hydrolase, and aromatic compounds. Moreover, several sourdough LAB are able to produce antimicrobial compounds, which can act as natural preservatives (Gerez et al., 2009). The predominant genera in sourdough are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella*, while *Lactococcus* and *Enterococcus* species are rarely found (Scheirlinck et al., 2007).

Species of fungi and bacteria belonging to the genus *Bacillus* are involved in the spoilage of bread and cause huge economic losses in the baking industry. There are currently a few bacteriocins that have been commercialized as natural biopreservatives for the control of spoilage and pathogenic organisms in foods. Bacteriocins are peptides (or proteins) produced by bacteria (including LAB) that have antimicrobial activity against other bacteria. The use of bacteriocin-producing LAB strains in food processing may be of considerable benefit to consumer health due to the lack of toxic effect and their ability to act as natural preservatives and thus delay spoilage (De Vuyst and Leroy, 2007; Macedo et al., 2012). Bacteriocins produced by pathogenic strains cannot be used in the food industry. As a consequence, researchers have tried to clone the genes for these bacteriocins into safer hosts (Yang et al., 2014); for example, Sanchez et al. (2008) cloned and expressed hiracin (a bacteriocin produced by *Enterococcus hirae*, which is known to be involved in food spoilage and nosocomial infections), into safer hosts. The authors successfully cloned and expressed this bacteriocin into *Lactococcus lactis*, *Lactobacillus sakei*, *Enterococcus faecium*, *Enterococcus faecalis*, and the yeast *Pichia pastoris*. They also succeeded in introducing and expressing hiracin in an additional two bacteriocin-producing LAB strains. These studies highlighted the possibility of using GM LAB strains with antimicrobial activity against food pathogens as potential starter cultures.

The main yeast species used in baking is *Saccharomyces cerevisiae*; however, other yeast strains have been isolated from sourdough including *Saccharomyces exiguus*, *Candida humilis*, *Candida krusei*, *Candida milleri*, *Issatchenkia orientalis*, *Pichia anomala*, *Pichia subpelliculosa*, and *Torulaspora delbrueckii* (Pulvirenti et al., 2004).

Baking utilizes fermentation, and during this process 95% of the sugars are converted into ethanol and carbon dioxide and 5% are converted into higher alcohols, carbonilic compounds, organic acids, and esters. Sugars (i.e., glucose, fructose, sucrose, and mannose) are provided by the flour (as a result of amylolytic degradation of the starch) and by the additives used in sweet doughs. In this manner the high sugar concentration acts as an osmotic stress factor. In plain

doughs the fermentable sugars are consumed within the first few hours, with maltose being the unique carbon source for the process. Therefore yeast strains used in baking must possess high fermentative abilities for different sugars, including maltose (Bell et al., 2001).

Maltose fermentation in *S. cerevisiae* is determined by the function of at least one MAL loci (*MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*), each of which is formed by three genes: two involved in maltose transport and one in regulation. These genes are highly expressed in industrial strains due to multiplication or mutations affecting the regulatory proteins (Novak et al., 2004). Maltose metabolism is regulated by maltose induction, glucose repression, and glucose inactivation. Improvement of maltose fermentation has been achieved by UV mutagenesis that led to the inhibition of *HXT1*, *HXT3*, and *HXT4* expression, which are genes that encode proteins involved in the uptake and transport of glucose from the growth medium (Salema-Oom et al., 2010).

Adaptive evolution has been used to select spontaneous mutants of a baker's strain of *S. cerevisiae* that demonstrated improved fermentative capacity of sweet dough, increased maltase and invertase levels, and a high stability during storage and cultivation (Rincón et al., 2001). Invertase from *Candida* spp. and *Saccharomyces* spp. are already used as food additives to hydrolyze sucrose in confectionary (Smith and Hong-Shum, 2003).

Mass mating between commercial *S. cerevisiae* strains used for sweet-dough and plain-dough leavening and selection under high-sugar stress conditions produced a novel strain with high osmotolerance, good maltose utilization, and high efficiency in leavening both types of dough (Higgins et al., 2001).

A problem for the bakery industry is maintaining yeast activity in frozen dough. Trehalose is a disaccharide composed of two α -glucose molecules that is found to accumulate in approximately 30% of yeast cells undergoing thermal stress. Kim et al. (1996) disrupted the acid trehalose (*ATH1*) gene to obtain a new yeast strain with augmented trehalose levels and with tolerance to dehydration, freezing, and toxic levels of ethanol. Baker's yeast strain cocultured with a mixture of soy peptides was also shown to possess improved tolerance to freeze-thaw stress and a higher stability of dough leavening abilities (Izawa et al., 2007).

Overexpression of the putative transcription factor involved in cell cycle regulation (*POG1*) gene in a diploid Japanese baker's yeast strain of *S. cerevisiae* produced a new strain with increased fermentation ability after freeze-thaw treatment (Sasano et al., 2013).

Proline is also involved in the cryoprotection of yeast cells due to its ability to form hydrogen bonds with free intracellular water, thus protecting the structure and function of the cell membrane during freezing. A self-cloning *S. cerevisiae* strain that was capable of accumulating proline was obtained by disrupting the proline oxidase (*PUT1*) gene and replacing the gamma-glutamyl kinase (*PRO1*) gene with alleles (Kaino et al., 2008).

A strategy for obtaining higher yeast biomass for baking consists of growing baker's yeast strains on novel cultivation media. For example, using a commercial compressed Italian strain [Pisano et al. \(2015\)](#) reported the isolation of a new nongenetically modified *S. cerevisiae* strain that was able to grow on cheese whey-derived medium supplemented with β -galactosidase from *Aspergillus oryzae*.

3.2 Beer

Making beer is one of the oldest activities of mankind. Early beer-making processes used sun-heated grains and produced a dark malt with a smoky flavor, while the drying of grains in ovens has allowed the production of pale ale. There are currently more than 70 types of beer, which can be classified according to their ingredients and the production process used. Yeasts represent a major component of the beer alongside grains, which provide the sugar substrate for fermentation, the flavor and body of the beer, the hop with high antimicrobial activity, and the water important for the mineral salts, which influences the quality and flavor of the beer.

Ales are produced by the fermentation of *S. cerevisiae*, a top-fermenting yeast. Recent studies showed that most top-fermenting yeasts are polyphyletic. While German and British yeast strains have nonrecombinant genomes, most other beer strains are a mixture of strains involved in wine, sake, beer and bread fermentation, which is probably as a result of independent domestication events ([Gonçalves et al., 2016](#)).

Lager is obtained at low temperatures (8–15°C) by *Saccharomyces pastorianus*, a bottom-fermenting interspecific yeast hybrid of *S. cerevisiae* and *Saccharomyces eubayanus*. Whole genome sequencing studies revealed that there is only limited genetic variation between the lager yeast strains and the ale yeast strains, with all lager yeasts descending from only two separate hybridization events ([Mertens et al., 2015](#)).

Genetic analyzes showed that protein synthesis and transcriptional regulation of genes involved in fermentation of the grains varies during the process ([Smart, 2007](#)).

One of the main aspects influencing beer making is the flocculation of yeasts. When *Saccharomyces* are cultivated on simple, complete media without external stress they are able to grow as unicellular yeast cells and multiply by budding; however, stress conditions (i.e., nutrient limitation) cause the yeast cells to switch to filamentous growth and form pseudohyphae. This process also triggers the activation of genes involved in the asexual aggregation of yeast cells into multicellular masses called flocs ([Gancedo, 2001](#); [Soares, 2010](#)). Flocculation is mainly important for the aroma of lager; however, early flocculation can reduce the maturation efficiency of the final product while late flocculation can produce beer that is cloudy and difficult to filter ([Dequin, 2001](#)). [Govender et al. \(2008\)](#) succeeded in controlling the flocculation process by cloning copies of the *FLO1*, *FLO5*, and *FLO11* genes from a haploid nonflocculent, noninvasive *S. cerevisiae* strain into different brewer's strains. The

genes were placed under the control of the alcohol dehydrogenase 2 (*ADH2*) promoter (which was induced by high ethanol concentrations) and the heat-shock protein 30 (*HSP30*) promoter (which was active during stationary growth phase) when the nutritional sources diminished and thus acting like stress factors. Since only yeast-derived DNA sequences have been used, the resulting strains were regarded as self-cloned and considered safe for further use.

During fermentation, pyruvate is converted to α -acetolactate (an intermediate of valine synthesis), which is further transformed to diacetyl through oxidative decarboxylation. The accumulation of diacetyl gives beer a butter or butterscotch flavor. To avoid this effect, beer is matured for 2–3 weeks to transform the diacetyl into acetone. Shortening the maturation process would be of interest as it would lead to a reduction in production costs. Enari et al. (1992) described a recombinant lager yeast strain that was used to accelerate the maturation of beer. This strain was produced by cloning a bacterial α -acetolactate decarboxylase gene, which catalyzes the decarboxylation of α -acetolactate in acetoin. Dulieu et al. (2000) proposed shutting down diacetyl formation using exogenous recoverable encapsulated α -acetolactate decarboxylase. Polaina (2002) mentioned reducing the expression of the *ILV2* gene (acetohydroxy-acid synthetase) and overexpressing the *ILV5* gene (acetohydroxy-acid reductoisomerase) involved in α -acetolactate synthesis. Wang et al. (2010) constructed a recombinant *S. cerevisiae* strain with accelerated maturation and high glucoamylase activity. Since no heterologous DNA sequences, bacterial sequences, or drug-resistance genes were introduced the new strains may be considered as safe for large-scale beer production.

Krogerus et al. (2015) generated interspecific larger yeast hybrids using a domesticated, flocculent *S. cerevisiae* strain and a strain of *S. eubayanus*. The hybrids inherited the characteristics of the genitors and were capable of accelerated fermentation and higher ethanol production (5.6% vs. 4.5% for the parental strains). Sato et al. (2002) improved fermentation at low temperatures by hybridizing a top-fermenting strain of *S. cerevisiae* and a cryophilic *Saccharomyces bayanus* strain.

The presence of hydrogen sulfite (H_2S) modifies the organoleptic properties of beer. Improvement strategies to reduce H_2S production and augment sulfite synthesis are also involved in flavor stabilization. However, EU and US regulations state that acceptable sulfite rates in beer must be below 10 mg/L (Guido, 2016). Hansen and Keilland-Brandt (1996) produced a beer with increased flavor stability by the partial or full elimination of *MET10* gene activity (which codes for the α -subunit of sulfite reductase). Other strategies to increase the production of sulfite or to accelerate the transformation of H_2S into cysteine and homocysteine involve the inactivation of the homoserine O-transacetylase (*MET2*) gene or overexpression of the O-acetylhomoserine sulfhydrylase (*MET25*) gene, respectively.

Glutathione has a major antioxidant role in the cell, is a product of cysteine metabolism, and is important for stability of beer flavor. Wang et al. (2008) obtained two self-cloning recombinant brewer's yeast strains with high glutathione and low diacetyl synthesis rates.

3.3 Wine

LAB strains have a long history in beverage fermentation. These bacteria are valued for their sensory qualities as they are able to modify flavors and their antimicrobial activity is important in the control of spoilage bacteria.

Yeast cells are responsible for alcoholic fermentation during the wine-making process. *Oenococcus oeni* and species of *Lactobacillus* and *Pediococcus* perform a secondary fermentation known as malolactic fermentation, whereby malic acid is converted to lactic acid. Malic acid produces a sour taste in comparison with lactic acid and therefore most wine producers have turned to malolactic fermentation to produce “softer” wines through the accumulation of lactic acid (Volschenkla et al., 2006).

LAB strains have also been investigated for their ability to produce enzymes involved in the synthesis of aroma compounds and their ability to produce antimicrobial compounds (i.e., bacteriocins) so as to reduce the concentration of chemical preservatives in wine. Genetic modification of an *O. oeni* strain was performed to investigate the impact of altering esterase gene expression on the wine aromatic profile during winemaking. Darsonval et al. (2016) cloned and expressed two esterase genes (*estA2* and *estA7*) in *O. oeni* ATCC BAA-1163. They succeeded in expressing a functional protein in *O. oeni* under enological conditions and this genetic modification led to a decrease in the concentrations of isobutyl acetate, hexyl acetate, isoamyl acetate, and ethyl hexanoate. This research has contributed to the understanding of esterase activity in the production of flavor compounds in wine.

The yeasts involved in wine making are highly diverse. On the surface of the grapes the main species (50%–75%) are *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Candida stellata* while only 1%–10% are *Pichia membranifaciens*, *T. delbrueckii*, and *S. cerevisiae*. During alcoholic fermentation most yeasts belong to *S. cerevisiae* and *S. bayanus*, or to *Candida*, *Pichia*, *Hansenula*, and *Brettanomyces* genera. At the end of the process, *Brettanomyces*, *Dekkera*, and *Zygosaccharomyces* yeast strains predominate. To be used for the production of wines the yeasts must present: (1) efficient fermentation; (2) augmented tolerance to temperature variations, high ethanol levels, and high sulfite concentrations; (3) production of extracellular enzymes (e.g., proteases, glucanases, and pectinases); (4) low levels of H₂S, acetic acid, and ethyl carbamate synthesis; and (4) killer resistance (Fleet, 2008). Therefore the criteria used to improve wine yeast varieties are correlated with these requirements.

The utilization of vectors and recombinant DNA technology to improve commercial wine yeast strains is rather difficult. The selection of transformants requires auxotrophic cells as hosts or the presence of another selection marker. To obtain auxotrophic mutants Hashimoto et al. (2005) used UV mutagenesis experiments on diploid sake *S. cerevisiae* strains. EMS was also used to obtain auxotrophs for a haploid derivative of a wine yeast strain (Steensels

et al., 2014). Other acceptable selection markers include the cycloheximide and geneticin resistance genes (Cebollero et al., 2007).

Recombinant DNA has been used to generate wine yeast strains that have an enhanced ability to liberate flavor compounds. For example, cloning of β -glucosidase genes from *A. oryzae* or *Candida*, *Hanseniaspora*, or *Pichia* genera, allowed the synthesis of terpenols from monoterpenyl glycosides found in wort and must (Verstrepen et al., 2006). Conversely, studies regarding the synthesis of carbon–sulfur lyase and alcohol acetyltransferase introduced the possibility of regulating gene expression to produce yeast strains with a high release of flavor-active molecules from grape juice. Rare mating allowed the generation of wine with a wider range of flavors and aromas following interspecific hybridization between an isolate of a commercial *S. cerevisiae* wine yeast strain and strains of *Saccharomyces paradoxus* and *Saccharomyces kudriavzevii* NCYC 2889 (Bellon et al., 2011).

Direct mating between *S. cerevisiae* and the cryotolerant species *S. kudriavzevii* and *S. bayanus* has been effective in generating strains with improved cryotolerance and flocculation for use in sparkling wines (Steensels et al., 2014). Augmented growth and fermentation rates at low-temperatures were obtained in commercial yeast strains provided by Lallemant Inc. (France) using EMS mutagenesis and adaptive evolutionary engineering under inositol limitation (López-Malo et al., 2015).

Adaptive evolution (using potassium chloride resistance as a stress inducer) along with breeding strategies were used by Tilloy et al. (2014) to improve *S. cerevisiae* Lalvin EC1118 (*Prise de Mousse*), a wine strain used as a base for Champagne. The resultant strains did not accumulate undesired compounds (i.e., acetate, acetaldehyde, or acetoin) and presented high survival rates under osmotic stress and glucose starvation due to enhanced glycerol production and reduced ethanol yields.

Another undesired compound produced during wine fermentation is H_2S . EMS mutagenesis was performed on an Australian commercial wine strain *S. cerevisiae* PDM, which already had low H_2S production. Cordente et al. (2009) isolated stable mutants for the genes *MET10* and *MET5* and the preliminary industrial-scale trials confirmed their possible use in the production of H_2S -free wine.

The presence of yeast strains with killer activity is of great importance for the fermentation process, especially for the production of sparkling wines, which undergo slow aging. Yeast autolysis contributes to this process as the dead cells release compounds, which are likely to influence the foam properties and wine flavor. Conversely, killer strains may be used as starters to inhibit the growth of undesired endogenous yeasts that may affect the quality of the wine. The killer phenotype in *S. cerevisiae*, *Hanseniaspora uvarum*, and *Zygosaccharomyces bailii* is determined by cytoplasmatic dsRNA virus-like particles and the mechanisms of action of the killer toxins have been extensively studied (Marquina et al., 2002). One

approach for improving wine making is to isolate and identify new species with killer activity. Ramírez et al. (2015) reported the complex characterization of *T. delbrueckii* strains isolated from spontaneous fermentations of grapes from Spain that were able to produce a new killer toxin (Kbarr-1). Protoplast fusion was used to transfer killer particles from the killer strain *S. cerevisiae* MTCC 475 to the distillery nonkiller strain *S. cerevisiae* HAU-1 (Bajaj and Sharma, 2010). Yap et al. (2000) reported that cocultivation of *Pichia anomala* and *Williopsis saturnus* var. *mrakii* strains was able to kill over 90% of 26 tested strains at pH 4–5. The coculture method was also used by Lombardi et al. (2015) with *S. cerevisiae* killer and sensitive strains during sparkling wine production. The yeast autolysate occurred rapidly and influenced the free amino acids, total protein, and polysaccharide content.

3.4 Dairy Products and Nutritional Additives for the Dairy Industry

Genetic modifications of LAB strains used for obtaining dairy products involve the release of acids, aromatic compounds, vitamins, enzymes, preservatives, natural polysaccharides, as well as resistance to bacteriophages. Genetically modified LAB strains belonging to the *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* genera have a long tradition in the fermentation of foods (Carminati et al., 2015; Lindgren, 1999).

LAB strains play an important role in dairy product manufacturing due to their ability to: (1) release proteases, lipases, and esterases, which are involved in the maturation of dairy products; (2) increase food safety through the release of bacteriocins and organic acids; (3) produce flavor compounds; (4) increase viscosity through polysaccharide secretion; (5) increase nutritional value through the delivery of short-chain fatty acids and vitamins (Florou-Paneri et al. 2013; Kongo, 2013).

Ripening of cheese is a complex process that is based on the breakdown of proteins, lipids, and carbohydrates (e.g., acids and sugars). The final concentration of these compounds dictates the texture and flavor of the cheese. Proteolysis involves the proteolytic cleavage of caseins into peptides and amino acids, which are further degraded to flavor compounds by other enzymes (Bockelmann et al., 2005). To accelerate the ripening of cheese, Joutsjoki et al. (2002) transferred genes encoding *PepN*, *PepC*, *PepX*, and *PepI* peptidases from a highly proteolytic *Lactobacillus helveticus* 53/7 strain into *L. lactis* DN209 using a food-grade cloning system. The results suggested that *L. lactis* transformants may play an important role in the proteolysis of maturing cheese by producing additional peptidase activity.

Besides their ability to ferment sugars a few *L. lactis* strains and various species of *Leuconostoc* are able to metabolize citrate. Citrate is present in fruit juices, milk, and vegetables and during fermentation it is transformed by LAB strains into aromatic diacetyl, acetoin, and butanediol. Diacetyl is an essential compound for the flavor of dairy products, such as buttermilk, butter, and cheese (An et al., 2007; Quintans et al., 2008). Following

the identification of citrate metabolism in LAB, a number of scientists have applied genetic modification techniques to increase the production of aroma compounds. Swindell et al. (1996) increased diacetyl production by inactivating the *aldB* gene encoding for α -acetolactate decarboxylase in *L. lactis* MG1363 using double-crossover homologous recombination. An et al. (2007) obtained the recombinant vector pJLECit containing the citrate permease gene from the plasmid pCM1 from *L. lactis* subsp. *lactis* biovar *diacetylactis* NIAI N-7. The vector was transferred to *L. casei* L-49-4 where citrate permease was expressed and the transformed cells were able to metabolize citrate to diacetyl and acetoin. The transformants were able to produce and maintain diacetyl and acetoin for a long time due to low activities of diacetyl and acetoin reductase.

Phage infection poses a problem for the dairy industry as it causes slow or incomplete fermentation, which has a direct consequence on the quality of the product. Research studies have furthered the understanding of phage defense mechanisms and recombinant DNA technologies have created bacterial strains with resistance to different phages (Marco et al., 2012). Studies on developing engineered systems were mostly performed on *Streptococcus thermophilus* and *L. lactis* strains due to their widespread use in dairy fermentation processes (Szczepankowska et al., 2013). Sturino and Klaenhammer (2004) created an *S. thermophilus* strain that was resistant to the κ 3 phage and two other Sfi21-type phages. The authors used antisense RNA expression vectors containing a putative primase gene and other genes associated with the Sfi21-prototype genome replication module. The results revealed a reduction in plaque efficiency between $0.8\text{--}3 \times 10^{-3}$.

Djordjevic et al. (1997) developed a novel bacteriophage protection system for *L. lactis* based on another phage resistance mechanism, the phage-triggered suicide system. The authors constructed a pTRK414H vector containing the *LlaIR* suicide cassette (three genes involved in restriction modification systems of lactococcal strains) under a phage Φ 31-specific trigger promoter. The vector was transferred to *L. lactis* NCK690 and the transformed cells were able to inhibit four Φ 31-derived recombinant phages at levels 10-fold greater than Φ 31. Transformed lactococcal cells were able to eliminate phages with the potential to evolve during fermentation by destroying their genome and killing the propagation host.

It is known that folate is an essential compound in the human diet; however, it cannot be stored in the human body. Folate deficiency may increase the risk of neural tube defects, cancers, and cardiovascular diseases. Wegkamp et al. (2003) performed genetic engineering in LAB strains to increase the folate concentration of dairy products. Not all LAB species are able to synthesize folate, therefore they cloned five essential folate biosynthesis genes from *L. lactis* MG1363 into the pNZ7017 vector. This vector was subsequently transferred into the nonproducing folate strain *Lactobacillus gasseri* ATCC 33323, after which the strain was capable of producing folate.

The number of yeast species identified in dairy products is limited. Their presence varies with the source of isolation: *Kluyveromyces lactis* and *Kluyveromyces marxianus* are seldom found in milk, yogurt, raw cheese, and kefir; *Kluyveromyces (Kazachstania) lodderae* is found in kefir; *S. cerevisiae* in raw cheese, yogurt, and kefir; *Saccharomyces boulardii* in milk, yogurt, and ice cream; *I. orientalis* in cheese, kefir, and yogurt; *Yarrowia lipolytica* and *Debaryomyces hansenii* in kefir and milk; and *Candida inconspicua* and *Candida maris* in kefir. Specific characteristics of these species include their: (1) ability to assimilate lactose (*K. lactis* and *K. marxianus*); (2) ability to assimilate lactic acid produced by the coexisting LAB and produce lipids and fatty acids required for the proliferation of microorganisms (*Y. lipolytica* and *D. hansenii*); (3) antimicrobial and antifungal activity related to the existence of the killer phenotype (*S. cerevisiae* and *K. lactis*); and (4) potential for producing probiotic compounds with benefits for human and animal health.

K. lactis was initially known as *Saccharomyces lactis* and *Zygosaccharomyces lactis* but gained the present denomination *K. lactis* (Dombrowski) in 1971. Molecular studies have shown a high degree of variability within the *K. lactis* species (Kurtzman et al., 2011). *K. lactis* is a Crabtree-negative yeast with the ability to perform aerobic glucose metabolism and assimilate lactose. Lactose enters a cell using lactose permease in the cell membrane and is converted by lactase (β -galactosidase) into glucose and galactose. Galactose is further transformed into glucose-6-phosphate through the Leloir pathway, which itself enters into glycolysis (Rubio-Teixeira, 2006). Lactase is produced from *K. lactis* on an industrial scale and is commercialized under the name Maxilact (DSM Food Specialties, The Netherlands) for the production of lactose-free dairy products. This represents a real benefit for lactose-intolerant individuals and assures a reduction of sugar in the diet (van Ooyen et al., 2006). Therefore improving lactase synthesis in *K. lactis* is of high importance. Dagbagli and Goksungur (2008) reported β -galactosidase (lactase) enzyme activity of 4218 U/g following optimization of culture conditions on synthetic medium containing 25 g/L of lactose, at pH = 7.35, agitation speed of 179 rpm, and an incubation time of 50.9 h.

K. marxianus also has numerous applications in the dairy industry. Although it shares some important characteristics with *K. lactis* (i.e., lactose assimilation), it also presents other interesting features, such as a high multiplication rate; the ability to ferment inulin (due to inulinase synthesis); the ability to produce ethanol, ribonucleotide, phosphatases, and aminopeptidase; and an enhanced thermo- and osmotolerance.

The synthesis of β -galactosidase (lactase) in *K. marxianus* requires reduced concentrations of lactose or galactose in the growth medium, as augmented intracellular galactose levels has an inhibitory effect due to the production of metabolic by-products from glycolysis (Gondim Martins et al., 2002). The influence of genetic and metabolic diversity on lactase production is obvious since the industrial process presents a high variability depending on the yeast strain, lactose uptake from the medium, enzyme thermostability, and environmental pH (Al-jazairi

et al., 2015; Fonseca et al., 2008). Strain-specific enzyme production was observed in the case of two *K. marxianus* CBS strains, 6556 and 712, with the latter requiring yeast extract to be supplemented in the growth medium (Rech et al., 1999). When working on the same strain (*K. marxianus* CBS 6556) Perini et al. (2013) found that a combination of parameters (820 mL L⁻¹ cheese whey, 14.36 g L⁻¹ of corn steep liquor, 31°C, and 24 h incubation) produced an optimal yield of enzyme.

Diniz et al. (2012) observed high ethanol rates in a strain isolated from dairy products (*K. marxianus* UFV-3) when it was cultivated on minimal medium (supplemented with 64 mmol L⁻¹ lactose) under hypoxic conditions. The explanation resides in the high gene expression levels of *LAC4*, *GAL7*, *GAL10*, and *RAG6* (pyruvate decarboxylase). Conversely, cocultivation of *K. marxianus* and *S. cerevisiae* cells immobilized on alginate beads on cheese whey powder medium, was found to enhance lactose fermentation and produce ethanol (Guo et al., 2010). This strategy overcomes substrate limitation since *S. cerevisiae* is not able to use lactose (and therefore cheese whey) as a carbon substrate. This problem is solved by using a coculture system with *K. marxianus*, which has the ability to grow on lactose and convert it to glucose, thus providing the main substrate for *S. cerevisiae* proliferation.

K. marxianus, *K. lactis*, *S. boulardii*, *S. cerevisiae*, *Saccharomyces dairensis*, and *I. orientalis* have received generally recognized as safe (GRAS) status from the Food and Drug Administration (FDA) and their enzymes have been permitted for use in various foods (van Ooyen et al., 2006). To assure their acceptance in the food industry the methods developed for their production have mainly aimed to optimize biomass and enzyme extraction.

K. marxianus can be used as a source of ribonucleases (flavor enhancers) by cultivating on cheese whey and performing autolysis (de Palma Revillion et al., 2003). Serine proteases are extracellular proteases involved in milk digestion. *K. marxianus* serine proteases are characterized by their high thermal and pH stability. Stergiou et al. (2013) reported high rates of synthesis by cultivation *K. marxianus* IFO008 on glucose-based medium supplemented with bovine serum albumin as nitrogen source.

Inulins are energy storage polysaccharides that are synthesized by various plant types. *K. marxianus* can produce cell-wall bound and extracellular inulinases (2,1-β-d-fructan fructanohydrolase), which convert inulin into fructooligosaccharides (FOS) (Vela Sebastião Fernandes and Jiang, 2013). FOS can be used as alternative sweeteners and are of real benefit for the human diet due to their low caloric value, their noncarcinogenic nature, their potential to be used by diabetic individuals, and their prebiotic action (stimulating the growth of probiotic microorganisms). Santos and Maugeri (2007) showed that sucrose concentration and temperature must be monitored when producing inulinase on an industrial scale using *K. marxianus* var. *bulgaricus*. The product was a mixture of glucose, fructose, sucrose, and FOS, for which the concentrations could be adapted according to the reactor parameters, thus making it suitable for use in various foods. An exoinulinase purified from *K. marxianus* with

thermal and pH stability was able to hydrolyze raw inulin from *Asparagus racemosus*. The syrup demonstrated a high fructose content (85.7%) and was thus of potential interest for industrial production (Singh et al., 2007).

3.5 Probiotics

Probiotics were defined by the WHO and Food and Agriculture Organization of the United Nations (FAO) as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). Most probiotic products include LAB that are recognized as safe for humans. Probiotic strains exert beneficial effects on human hosts by: (1) liberating health-enhancing bioactive compounds, which improve digestion and absorption in the intestinal tract; (2) maintaining the balance of the gut microbiota (e.g., colonizing gut epithelium and releasing antimicrobial compounds); (3) lowering cholesterol levels; (4) neutralizing dietary carcinogens; and (5) providing immunomodulatory activity (Florou-Paneri et al., 2013; Pelinescu et al., 2011). Probiotic strains that are currently on the market usually exert one or two of the aforementioned effects. Therefore researchers have tried to fill the gap in the probiotic activity spectrum by improving LAB strains using genetic engineering. These genetically modified probiotic strains can be used in healthcare, especially for the prevention and treatment of digestive diseases and food-borne illnesses. Research on the interactions between gut microbes, pathogenic strains, and the immune system has led to the identification of key regulatory molecules that are involved in the development of different digestive diseases. Through genetic engineering it has become possible to increase the expression of these bioactive compounds using probiotic strains (Saber et al., 2011; Steidler and Neiryneck, 2008).

Some of the targets for the genetic modification of probiotic strains have included host gut immunomodulation, antimicrobial compound release, vitamin synthesis, short-chain fatty acids and polysaccharides with prebiotic effects, adhesins, and digestive enzymes (Kullen and Klaenhammer, 2000).

Inflammatory bowel disease is a group of intestinal disorders caused by prolonged inflammation of the digestive tract due to an impaired immune response to the gut microbiota. The maintenance of intestinal homeostasis requires a balance between gut microbiota and immune cells; deregulation of these immunological interactions leads to inflammation. Several studies have generated genetically modified probiotic strains that produce antiinflammatory effects (de Moreno de LeBlanc et al., 2015; Martin et al., 2014); for example, *Lactococcus lactis* LL-Thy12 strain replaced the thymidylate synthase gene with a synthetic sequence encoding mature human interleukin-10. This food-grade strain was able to secrete antiinflammatory cytokines (IL-10) and preclinical studies have shown it be effective in the treatment of colon inflammation (Braat et al., 2006).

Mohamadzadeh et al. (2011) demonstrated that alteration of lipoteichoic acid, a cell surface component from *Lactobacillus acidophilus* NCFM, downregulated IL-12 and TNF α and

significantly enhanced IL-10. This could also represent a potential strategy for the treatment of inflammatory bowel disease.

3.6 Meat Products

Bacterial species, such as *Lactobacillus sake*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* together with *Micrococcus* and *Staphylococcus* are used as starter cultures in the fermentation of meat products. The starter cultures release flavor compounds during fermentation to provide meat products with specific tastes. The main reactions involved in the generation of flavor compounds are phospholipid hydrolysis followed by free fatty acid oxidation and esterification, carbon source degradation to produce organic acids, and peptide and amino acid conversion into alcohols, aldehydes, and acids (Boyacioglu et al., 2010).

LAB bacteria play an important role in the production of sausages. During fermentation, LAB are able to produce various organic acids that are able to decrease the pH, which in turn causes protein coagulation, inhibition of microbial strains involved in food spoilage, and the generation of an acid taste (Hammes and Hertel, 1996). An example of improved starter culture for meat products using genetic engineering technique was described by Hammes and Hertel (1996) who made two lactobacilli strains with antimicrobial activity against the food pathogen *Staphylococcus aureus*. They cloned and expressed the genes coding for catalase (*katA*) and lysostaphin (*lys*) in *L. sake* LTH673 and *L. curvatus* LTH 1432 and found that the transformed strains acquired the potential to kill staphylococci in fermenting sausages.

3.7 Fermented Vegetable Products

Vegetables harbor many microorganisms including LAB strains; however, these are present in lower numbers than spoilage microorganisms (e.g., *Pseudomonas*, *Erwinia*, and *Enterobacter* genera). LAB strains rapidly increase in number during fermentation due to their resistance to high NaCl concentrations. They release organic acids (especially lactic acid), inhibit the development of undesirable microorganisms, and extend the shelf life of the product (Breidt et al., 2013). The release of flavor compounds also increases the overall quality of these products. Various LAB species (*L. plantarum*, *Leuconostoc* sp., *Pediococcus*, and *Lactococcus*) are used in the fermentation of vegetables, such as pickles, olives, and cabbage (Boyacioglu et al., 2010).

The advantages of fermented vegetables are that they are microbiologically safe, they could represent a probiotic source, they have high nutritional values, they have appealing sensory characteristics, and they can be stored for extended periods. However, current vegetable fermentation practices are faced with some problems, such as the requirement for improved

starter cultures to enable fermentation at low salt concentrations, phage infections, a need to enhance the antimicrobial activity against microbial strains involved in spoilage, and a desire to increase the nutritional value and flavor (Breidt et al., 2013). All these problems could be solved using genetically engineered microbial strains.

3.8 Other Industrial Enzymes and Food Additives

Certain microbial strains are able to produce valuable substances that can be used as food additives (i.e., vitamins, polysaccharides, amino acids, flavors, enzymes, and organic acids). Some of these food additives are currently obtained with the help of GMMs, which allow precious substances to be obtained at a more competitive price than conventional industrial methods.

3.8.1 Urease

LAB strains have a long history in food processing and most members are known to be safe for human health. Food manufacturers found that they can also be attractive cell factories for the production of many important food ingredients (additives) including bacteriocins, lactic acid, vitamin B, and urease.

Urease produced by *Lactobacillus fermentum* facilitates the hydrolysis of urea to ammonia and carbon dioxide. The enzyme is used in alcoholic beverages (especially in wine production) to inhibit the formation of ethyl carbamate, a carcinogenic compound that is spontaneously produced by the reaction between urea and ethanol (Kakimoto et al., 1990).

Since urea is considered to be the main precursor of ethyl carbamate in alcoholic beverages, producers added ureases to their beverages to decrease urea levels in the final product. Urease extracted from *L. fermentum* was found to be active in acidic conditions that were similar to those used in the fermentation of alcoholic beverages. Visser et al. (1997) cloned the genes involved in urease synthesis from *L. fermentum* and expressed them in *S. cerevisiae* to enable the yeast cells to degrade urea under acidic conditions; however, the activity of the yeast urease was very low and probably required the presence of other LAB genes to assemble an active enzyme.

3.8.2 Lipases

Lipases (triacylglycerol acylhydrolases) are serine hydrolases that catalyze the hydrolysis of triacylglycerides and, under certain environmental conditions, the reverse reaction of esterification, transesterification, and interesterification. Industrial lipases are esterases that can act on fats and oils to form glycerol and fatty acids (Saxena et al., 1999).

Of all the known lipases, those synthesized by microorganisms (e.g., bacteria, yeasts, and fungi) have the largest applicability due to the existence of simple technologies and

their broad spectrum of catalyzed reactions. Current uses of lipases in the food industry include: (1) as taste enhancers, (2) the preservation of baking products; (3) as aroma enhancers in beverages; (4) the hydrolysis of fats in dairy products and cheese maturation; (5) the improvement of lipid hydrolysis in oils and fats for mayonnaise and sauces; (6) fat processing and removal from fish and meat products; and (7) cocoa butter and margarine production.

Yeast species intensively studied for lipase production include *Candida albicans*, *Candida rugosa* (*C. cylindraceae*), *Geotrichum candidum*, *Trichosporon fermentans*, and *Y. lipolytica*. *C. rugosa* (*C. cylindraceae*) contains multiple lipases encoded by the *LIP1*–*LIP7* genes. Some of the lipases are isoforms with different thermal stabilities and substrate specificities. Isoforms appear due to modifications in gene expression, partial proteolysis, and posttranscriptional processes. Currently there are Lip1, Cel1, and Cel3 lipases encoded by the *LIP1* gene; Lip2, Lip3, and a mix of four isoforms from lipase B (Lopez et al., 2004). Lip1 is the major *C. rugosa* lipase used in industrial reactions, followed by Lip2 and Lip3 (0%–25%) (de Maria et al., 2006). *C. rugosa* lipases are currently being commercially produced and are active in higher fats and oils as they are used in the manufacture of cheese, egg products, and food oils (Smith and Hong-Shum, 2003).

Intensive research has been conducted to improve *C. rugosa* enzymes so they can be efficiently used in industry. The main strategies used for this purpose have included the engineering of substrate parameters, mutagenesis and selection of new strains, protein engineering, and improved enzyme characteristics (i.e., thermal and solvent stability and regio-, stereo-, and enantioselectivity) (Bassegoda et al., 2012).

The synthesis of lipase isoforms depends on the existing carbon and nitrogen sources; for example, oleic acid suppresses the expression of *LIP1* but induces the expression of *LIP3*. *LIP2* and *LIP4* are expressed in the presence of oleic acid and olive oil, while *LIP5* expression is active under the same conditions, even in the presence of a suppressing substrate (i.e., glucose). *LIP4* is also induced by the presence of Tween 20 (Akoh et al., 2004) and both Tween 20 and Tween 80 are able to induce lipase gene transcription. High lipolytic activity was observed when *C. rugosa* was grown in YNB media supplemented with 2.5% Tween 80, or yeast extract medium with 1% olive oil (Csutak et al., 2015). Fadiloglu and Erkmén (2002) also reported high lipase synthesis for *C. rugosa* grown in a peptone and yeast extract medium containing olive oil and Tween 80, while glucose, glycerol, and lactose inhibited lipase production.

Site-directed mutagenesis techniques have also been used to improve the characteristics of *C. rugosa* lipases. The 19 CUG codons in *LIP4* were replaced with serine codons and the recombinant gene was expressed in *P. pastoris* host cells (Tang et al., 2001). The resultant lipase had similar activity to the commercial enzyme, showed increased thermal stability, higher lipase activity toward unsaturated and long-chain lipids, and higher esterase activity toward C₁₆ and C₁₈ esters. Site-directed mutagenesis was also

used to convert nonuniversal serine codons (CTG) in *LIP2* into universal serine codons (TCT) (Lee et al., 2002). The recombinant *LIP2* gene was overexpressed in *P. pastoris* and the secreted enzyme was found to prefer short-chain fatty acids (butyric acid) for esterification, most probably due to differences occurring during glycosylation or due to the additional N-terminal peptide. The new enzyme could be used in the production of flavors for butter or cheese.

Zhang et al. (2016) produced a *C. rugosa* lipase with enhanced thermal stability using the active center stabilization strategy. They constructed a mutant *LIP1* library and selected the thermoresistant (60°C) mutants using a three-tier screening protocol.

Y. lipolytica lipases were first described in 1948 by Peters and Nelson. Since then more than 16 genes involved in lipase synthesis have been identified. Some *Y. lipolytica* lipases are localized intracellularly while others are released to the environment. However, there are still many questions regarding lipase gene expression in *Y. lipolytica*. Comparative analysis of microarray data and proteomic studies showed that *LIP2* was induced in media containing oleic acid while *LIP11* and *LIP13* were induced in media containing glucose (Fickers et al., 2011). Lipase production has also been noted in the presence of olive oil, oleic acid, sunflower oil, tributyrin, and Tween 80 as the nitrogen source is also an important factor (Gonçalves et al., 2014; Pignede et al., 2000).

Y. lipolytica extracellular lipase production was improved by mutagenesis of N-methyl-N'-nitro-N-nitrosoguanidine in media containing olive oil and organic nitrogen substrates (Destain et al., 1997; Galvagno et al., 2011).

High *Lip2* synthesis rates were obtained after UV mutagenesis (Darvishi, 2013). Recombinant DNA technology was also used to overexpress *Lip2* in *Y. lipolytica* containing a novel expression vector. The vector was devoid of bacterial DNA but contained a sequence derived from the retrotransposon Ylt1, which permits multiple integrations of the expression cassette containing the cloned gene (Pignede et al., 2000). Many attempts have been made to improve the thermal stability of *Lip2* in the hope of extending its practical applications. Wen et al. (2012) proposed three protein engineering strategies for this purpose: error-prone PCR-based directed evolution, semirational design, and site-directed mutagenesis.

Lipases from *K. marxianus* have provided a new research interest due to the GRAS status of this species. An increase of extracellular lipase synthesis (18-fold) was obtained after a 65 h fermentation in the presence of 0.5% olive oil (Stergiou et al., 2012). Tributyrin and urea or yeast extract and peptone were also found to induce lipase production (Csutak et al., 2016; Deive et al., 2003). Lipases hydrolyzed tributyrin to release butyric acid and this finding has potential application in the production of probiotic compounds since recent studies showed that butyric acid inhibits the growth of *Helicobacter pylori* (Yonezawa et al., 2012), has a major positive impact on energy metabolism in the host, and is involved in gene regulation (Kasubuchi et al., 2015).

3.8.3 Flavors and aromas

Y. lipolytica produces lipids, fatty acids, and citric acid, which is used to produce flavors in the food industry. Redox and metabolic engineering were used to produce new strains of *Y. lipolytica* with increased electron-donor characteristics of the cell membrane. These strains were isolated on media containing methyl ricinoleate, which is converted into ricinoleic acid and subsequently to gamma-decalactone, which provides a peach-like aroma to fermented foods (Wache et al., 2006). *Y. lipolytica* cells were also used as hosts for the expression of lipid-derived flavor molecules, such as aldehydes and alcohols (so-called “green notes”). Cloning the gene for fatty acid hydroperoxide lyase from green pepper Bourel et al. (2004) obtained 350 mg C6-aldehyde/L of reaction medium.

Citric acid is a main additive in the food industry as it can be used as a flavor, an antioxidant, a preservative, an acidulant, or an emulsifier. Yeast strains that contain citric acid include *Candida catenulata*, *Candida guilliermondii*, *Candida intermedia*, *Candida paratropicalis*, *Candida parapsilosis*, *Candida zeylanoides*, *Rhodotorula mucilaginosa*, *Rhodotorula gracilis*, *Y. lipolytica*, and *P. anomala*. Citric acid is produced by fermentation and the efficiency of the process depends on the yeast species, the carbon substrate (e.g., glucose, ethanol, edible oils, molasses, starch hydrolysates, glycerol, and *n*-alkanes), the nitrogen sources (e.g., yeast extract, urea, and ammonium chloride), the pH, and the presence of divalent metal ions (e.g., zinc, copper, and magnesium) (Yalcin et al., 2010). Citric acid production can be improved by mutagenesis or chemical agents (i.e., ethyl methane sulfonate and nitrous acid are the most effective) (West, 2016; Yalcin, 2012). The FDA has approved strains of *C. guilliermondii* (sec. 173.160) and *Y. (C.) lipolytica* (sec. 173.165) for the production of citric acid.

3.8.4 Sweeteners

Sorbitol is a low-calorie sugar alcohol that is used as sweetener in the food industry and is usually obtained by the hydrogenation of d-glucose in a metabolically engineered strain of *L. plantarum*. Ladero et al. (2007) overexpressed two sorbitol-6-phosphate dehydrogenase genes (*srlD1* and *srlD2*) in an *L. plantarum* strain deficient for both l- and d-lactate dehydrogenase activities, obtaining a conversion efficiency of 61%–65% from glucose.

3.8.5 Bacteriocins

Interest in bacteriocins has grown because some of these peptides are used as natural preservatives to enhance food safety. Bacteriocins are also used in a variety of food products (e.g., cheese and canned food), as an adjuvant for starter cultures, and they can be incorporated into polymers for packaging to decrease the number of food-borne pathogenic bacteria (Alvarez-Sieiro et al., 2016; De Vuyst and Leroy, 2007).

Bacteriocins produced by LAB strains have many attractive qualities that make them suitable for use as food preservatives: (1) they do not have cytotoxic and allergenic effects;

(2) they are inactivated by digestive enzymes of the gastrointestinal tract; (3) they are thermoresistant and are able to maintain their antimicrobial activity after sterilization; and (4) they have antimicrobial activity against bacteria involved in food spoilage (e.g., *Listeria monocytogenes*, *S. aureus*, *Bacillus cereus*, and *Clostridium* sp.) (Juodeikiene et al., 2012). The first bacteriocin commercialized as a natural preservative for the food industry was nisin (E234). This bacteriocin is produced by the GRAS microorganism *L. lactis* and has been approved as a food additive in over 50 countries. Other bacteriocins (e.g., pediocin, sakacin, plantaricin, carnocin, and enterocin) produced by LAB have received approval for use in the food industry.

3.9 GMMs: Harmful or Helpful?

The human population is estimated to reach 10 billion by the end of the 21st century and this expansion will require efficient management of natural resources (Bongaarts, 2009). The main concerns of the food industry manufacturers are covering market demands and increasing the quality of food products. Food manufacturers have recently released new or improved products into the market to satisfy consumer demands and to defeat competitors. To meet the future food demands extensive research must be performed to develop and improve food technology. Applying modern biotechnology techniques in the food industry could enable the improvement of food production (Sybesma et al., 2006). Genetic engineering techniques, such as recombinant DNA and the use of the resultant GMOs and GMMs could represent a viable alternative for the food industry due to their relatively low production costs and their potential to produce food products with high organoleptic properties. However, the general public has major concerns about the impacts of GMOs and GMMs on human health and the environment.

To be used in foods, GMOs and GMMs must pass several extensive tests and meet legal regulations. In 1997 the European Novel Food Regulation (EC 258/97) was launched, stipulating that foods and food ingredients derived from GMOs must not present a danger for consumers nor mislead the consumers, and must present high equivalent properties to similar products that are considered as safe. The European Parliament and Council later established [Regulation (EC) No 178/2002] the European Food Safety Authority (EFSA), which provides scientific advice and communicates the risks to the public regarding matters of food safety. According to the EFSA, the risk assessment for GMO includes molecular studies of the newly created proteins and evaluation of potential toxicity, allergenicity, and environmental impact. The authorization released by the EFSA after evaluating a GMO is valid for 10 years, after which a reassessment is required. There are numerous EU regulations and directives concerning the contained use of GMMs in food production (Regulation EC No 1829/2003), the release of GMOs into the environment (Directive 2001/18/EC), the safety assessments that a GMO must undergo before being placed on the market within the community

(Regulation EC 1829/2003), and regulations on the applications for authorizations of GM food and feed (Implementing Regulation 503/2013).

Similar legal mechanisms regarding food safety are also active in other parts of the world. The United States FDA produces guidance documents and regulations for foods, food additives, added substances, and dietary supplements. Although the FDA does not require special labelling of products containing GMOs or GM products, they announced a guidance for “Voluntary labeling indicating whether foods have or have not been derived from genetically engineered plants” including plant-derived food products or ingredients made with or without bioengineering. The binational government agency Food Standards Australia New Zealand is responsible for the Australian and New Zealand Food Standards Code comprising the requirements for substances added or present in food, contaminants and residues, microbiological limits and processing requirements, labelling, and GM food (Standard 1.5.2 – Food produced using gene technology). In Japan the use of GMO in food is based on The Cartagena Protocol on Biosafety to the Convention on Biological Diversity and is more relaxed depending on whether the food directly contains GMOs.

Despite these constraints, it seems that the regulations are more flexible concerning the use of foods and feeds that have been genetically improved via alternative techniques (classical breeding or random mutagenesis) using microbial strains (Sybesma et al., 2006).

Of the huge amount of studies conducted to improve microbial strains for food production, only a few have been approved for industrial use and these include the GRAS microbial species. Chymosin is used for dairy processing and was produced for the first time in 1988 by Gist-Brocades. It has since been commercialized as Maxiren and is currently trademarked by DSM (The Netherlands). This strain was derived via fermentation from a nonpathogenic and nontoxigenic strain of *K. marxianus* var. *lactis*, and contains the cloned prochymosin gene from calf stomach (rennet) (Han, 2004). In 1990 the United Kingdom approved this strain for use in food and the first engineered baker's yeast strains were able to produce carbon dioxide more quickly than the conventional strain due to derepressed maltose-utilizing enzymes (Nevoigt, 2008). During the 1990s the British government also approved the use of a dextrin-assimilating brewer's yeast strain containing the extracellular glucoamylase (*STA2*) gene from *S. cerevisiae* var. *diastaticus*. This strain showed improved ethanol production without alteration of the fermentation efficiency or the quality of the beer (Dequin, 2001). In 2001 the Japanese government accepted a recombinant cerulenin-resistant mutant sake yeast strain (FAS2-1250S) that was able to produce ethyl caproate, a flavor component important for the quality of sake (Akada, 2002). In 2003 Lasaffre Yeast Corporation received the GRAS notification for their malolactic fermentation-efficient recombinant yeast strain, ML01. This strain was produced by cloning in the industrial wine strain 592 (Lasaffre collection), the gene for malate permease from *Schizosaccharomyces pombe*, and the malolactic enzyme from *O. oeni*. The recombinant strain is currently used for winemaking in the United States and

the Republic of Moldova (Cebollero et al., 2007). The modified wine yeast strain ECMo01 was notified as GRAS in Canada (2006) and is used to reduce ethyl carbamate in fermented beverages. Ethyl carbamate is a by-product of fermentation and results from the combination of urea (from yeast cells) and ethanol (from the environment). The ECMo01 strain is the commercial *S. cerevisiae* wine strain Davis 522 carrying a recombinant urea amidolyase gene (*DURI,2*) from *S. cerevisiae* that results in the effective degradation of urea within the yeast cells (Pretorius et al., 2012). *S. cerevisiae* GRN 422/2012, which is able to reduce acrylamide production in grained and vegetable-based foods, coffee, and coffee substitutes, was obtained by cloning the periplasmic L-asparaginase II (*ASP3*) gene from *S. cerevisiae* S288C into a domesticated *S. cerevisiae* industrial bread yeast strain in 2012.

There are many applications for food enzymes derived from microbial-modified strains that are currently pending approval according to the Article 17(2) of Regulation (EC) No 1332/2008. These include α -galactosidase from *S. cerevisiae*, triacylglycerol lipase and hexose oxidase from *Hansenula polymorpha*, and phospholipase C from *P. pastoris*. The list also comprises new enzymes for obtaining or processing improved foods, such as urease from *L. fermentum*, membrane alanine aminopeptidase from *L. lactis*, and alternansucrase from *Leuconostoc citreum* (EU Food enzyme applications, 2016).

Legal matters concerning the use of GMMs in foods are strongly influenced by scientific research, public opinion, and lobbying of industrial groups from the food industry. However, it seems that the limiting factor for GMM use is consumer perception and concern regarding the effects on human health, environmental pollution, and biodiversity loss. As a result, numerous nongovernmental organizations have attempted to debate the latest issues relating to this topic. To develop a deeper understanding of GMOs and aid their acceptance, the food industry and governmental organizations should provide more information on the subject and develop trustworthy risk management processes. For example, it would be important to know that the microbial strains used in industry are selected for use under controlled environmental conditions and therefore (most probably) they are not able to compete with natural strains. Genetic experiments aim to induce or develop economically useful characteristics that are of no real benefit to the host strain, which could provide an evolutionary advantage over wild strains. However, since GMM-derived foods and food additives must exhibit similar characteristics to their conventional counterparts, and are subject to thorough examinations prior to their approval, it is not expected that their consumption would lead to health problems (Verstrepen et al., 2006). Moreover, although enzymes produced by GMMs have been on the market for many years in many countries, there are no medical reports that suggest they cause health problems. In fact, allergenic tests revealed that there are no differences between these enzymes and those obtained from non-GMMs (Aberer et al., 2001). Finally, according to Frewer et al. (2004), consumer hesitation in accepting GMOs and GMMs in foods is mostly due to poor communication with the food safety authorities and their failure to better integrate the public opinion into risk management analysis.

4 Conclusions

The food industry is constantly trying to explore new ways to improve product qualities to satisfy consumer requirements. The development of new microbial strains as starter cultures that are able to release valuable compounds can limit the need to add compounds into food products. Specific improvements are often required for starter cultures and sometimes these new features cannot be achieved using classical techniques (e.g., mutagenesis or adaptive evolution). Thus recombinant DNA technology can be ideal for improving microbial performance due to its high precision. Even though (from the scientific point of view) most GMMs do not represent a real danger in foods, the main problems in introducing them to the market are related to the restrictive food legislations and consumer reticence. However, a limited number of GMMs are currently used in the food industry, most of them being yeast strains. The main players in food production and marketing are searching for new solutions by cooperating with governmental organizations and researchers and developing additional alternative techniques, which could represent a viable solution for genetic improvement of food related microorganisms. However, it is obvious that establishing new communication channels with consumers and implementing trustworthy risk management processes could help to develop a deeper understanding of GMMs and (most probably) assure a better public reception.

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Techniques for Production and Quality Assessment of Genetically Modified Foods

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1 Introduction

The introduction of genetic engineering in agriculture and food production has meant that food safety has become a prominent issue. Genetic engineering (the targeted manipulation of genetic material) and nontargeted, nontransgenic methods (i.e., chemical mutagenesis and breeding) are some of the methods used to modify the genetic composition of plants, animals, and microbes. Such genetically engineered organisms are also referred to as genetically modified organisms (GMOs). Genetic engineering involves the insertion of a desired gene (from any species) in a food crop or food product. Although recombinant DNA technology is not inherently hazardous, the inserted genes may produce hazardous substances. Nongenetic engineering methods include embryo rescue, in which embryos of plants and animals produced from interspecies gene transfer or crossing are placed in a tissue culture environment to complete development. Other methods include somatic hybridization, in which the cell walls of plant cells are removed so that “naked” cells can hybridize and mutagenize. The development of these methods has drastically increased the spectrum of techniques that can be employed to enhance food production. However, like all other techniques for genetic manipulation, they also carry the potential to introduce unintended compositional changes that may have harmful effects on human health.

Humans mainly rely on plants to satisfy their food requirements thus health issues related to genetically modified (GM) crops are a major concern. Tobacco was the first GM crop to be produced in 1983, followed by the Flavr Savr tomato, and this opened up a completely new arena in the field of crop biotechnology. GM crops cater to the needs of the ever-increasing human population. These crops are resistant to diseases and environmental stresses, and exhibit increased nutritional values and shelf lives (i.e., Golden Rice). The first commercial production of GM crops was approved in 1996 primarily in the United States, Canada, Argentina, and Australia. Although hundreds of different GM plants have been partially or fully developed, a few traits dominate the commodity market, have been bred into genetically diverse varieties, and

have been widely adopted in many countries. Approved GM crops were grown on approximately 160 million ha in 29 countries in 2011 ([Goodman et al., 2013](#)). Scientists are currently developing edible vaccines using bananas, potatoes, soybean, rice, and legumes. These vaccines are not only cost effective but they are also easy to administer and store. However, the safety and ethical concerns regarding these crop plants remains an issue of debate. The safety assessment of GM foods generally involves determining the stability of the inserted genes, tendency to provoke allergic reactions, unintentional effect caused due to gene insertion, direct health effects, and specific components thought to possess nutritional, as well as toxic properties.

The aim of the food safety assessment is to determine if the GM crop food material is substantially equivalent to the non-GM counterpart in terms of food safety, or if it is unacceptably more hazardous under common growth and processing conditions. It has long been recognized that no food can conceivably be judged as 100% safe or without risk to humans. Approximately 4% of consumers in the United States, the European Union, and Japan collectively experience food allergies due to the development of specific IgE antibodies to one or more proteins present in the foods. Any GM variety would be expected to cause similar food allergies to their non-GM counterpart and pose the same risks for the same consumers.

New GM crops are tested more rigorously under regulatory scrutiny than crops modified by intentional and natural mutagenesis or those produced by crossing with wild relatives to introduce new traits (i.e., disease resistance and drought tolerance).

This chapter describes the types of GM foods, the modification methods, and the various techniques, as well as approaches for assessing the risks that GM foods pose to human health and the environment.

2 Types of GM Foods

Genetic modification of food is not new. Several GMOs have been developed for use in research, pharmacotherapies, and agriculture. All GMOs are developed with more added features and some of them are widely used as food. In 1997–99, GM ingredients formed two-thirds of all processed foods in the United States, which was fueled after a ruling by the Supreme Court, which stated that the patenting of life forms was allowed for commercialization. Since then myriads of applications for GMOs have been filed worldwide. In 2003 it was estimated that countries that grew 99% of the global transgenic crops were Argentina (21%), the United States (63%), Canada (6%), Brazil (4%), China (4%), and South Africa (1%) ([Langtree, 2009](#)).

Food crops and animals have traditionally been changed through selective breeding. Although genes can be transferred using this technique, the scope of exchanging genetic material is much wider when using genetic engineering. The most common GMOs are crop plants; however, the technology has been extended to almost all forms of life. Different types of GM foods are discussed later and a summary is presented in [Table 7.1](#).

Table 7.1: Different types of GM foods (Simó et al., 2014).

GM Crops	Donor Species	Genetic Modification	Phenotype
Rice	<i>B. thuringiensis</i>	<i>Cry1Ab</i>	Insect resistance
	<i>Zea mays</i>	<i>C1</i> and <i>R-S</i>	Flavonoid production
	<i>Oryza sativa</i>	<i>YK1</i>	Stress tolerance
	<i>O. sativa</i>	<i>RCH10</i> , <i>RAC22</i> , and <i>B-RIP</i>	Antifungal activity
	<i>Escherichia coli</i>	<i>LysC</i> and <i>SDH</i>	Nutritionally enhanced
Maize	<i>Z. mays</i>	<i>Rpd3</i>	Seed development
	<i>B. thuringiensis</i>	<i>Cry1Ab</i>	Insect resistance
	<i>Z. mays</i>	<i>Zmp syl</i> , <i>PacrtI</i> , <i>Glycb</i> , and <i>ParacrtW</i>	Nutritionally enhanced
Alfalfa	<i>Nicotiana tabacum</i>	<i>PAL2</i>	Nutritionally enhanced
Wheat	<i>Ustilago maydis</i>	<i>Chit/Gluc</i> and <i>RIP</i>	Fungal resistance
	<i>Triticum aestivum</i>	<i>Glu-A1</i> and <i>Glu-D1</i>	Nutritionally enhanced
Soybean	<i>A. tumefaciens</i>	<i>CP4 EPSPS</i>	Herbicide tolerance
	<i>N. tabacum</i>	<i>ASA2</i>	Nutritionally enhanced
Potato	<i>Aureobasidium pullulans</i>	<i>W2</i> , <i>FK</i> , <i>Mall</i> , and <i>SamDC</i>	Starch biosynthesis, leaf morphology, and ethylene production
	<i>Cynara scolymus</i>	<i>1-SST</i> and <i>1-FFT</i>	Inulin synthesis
	<i>Symphytum tuberosum</i>	<i>AGPase</i> , <i>StcPGM</i> , and <i>StpPGM</i>	Altered starch composition
	<i>A. tumefaciens</i>	Potato virus Y	Virus resistance
	<i>A. thaliana</i>	<i>DREB1A</i>	Stress tolerance
	<i>Saccharomyces cerevisiae</i>	<i>TPS1</i>	Drought resistance
	<i>A. pullulans</i>	<i>W2</i>	Waxy phenotype
	<i>E. coli</i>	<i>Ent/CpmsB</i>	Salicylic acid producing
	<i>Thaumatococcus daniellii</i>	<i>Thaumatocin-II</i>	Sweet flavor
	<i>E. coli</i>	<i>Asn A</i>	Growth enhanced
Tobacco	<i>RBDV</i>	Virus movement protein	Virus resistance
Cucumber	<i>E. coli</i>	<i>Adh</i>	Abiotic stress
Lettuce	<i>B. amyloliquefaciens</i>	<i>GluB</i> and <i>ChGP</i>	Antifungal activity
Raspberry	<i>Mentha × piperita</i>	<i>MFS gene</i> and <i>DXR</i>	Essential oil content
Grapevine	<i>Populus trichocarpa</i>	<i>hipl-SOD</i>	Superoxide production
Barley	<i>Carica papaya</i>	<i>5S-1</i>	Virus resistance
Peppermint	<i>A. tumefaciens</i>	<i>Bar</i>	Herbicide tolerance
Poplar	<i>A. tumefaciens</i>	<i>LBA4404</i>	Improved texture and color
Papaya	<i>Richadella dulcifica</i>	<i>Miraculin</i>	Sweet flavor
Cabbage	<i>A. thaliana</i>	<i>AtKXK1</i>	Altered carbohydrate metabolism
Tomato	<i>Vitis vinifera</i>	<i>Stilbene synthase</i>	Resveratrol synthesis
	<i>Z. mays</i>	<i>LC1</i> , <i>C1</i>	Increased flavonol content
	<i>E. coli</i>	<i>DXS</i>	Increased carotenoid content

2.1 Cotton

Cotton is considered as a food crop because the oil obtained from its seeds can be consumed and it forms an important part of animal feed. Cotton production is susceptible to insects and pests and cotton bollworms cause damage of more than US \$300 million per year. In India more than US \$660 million is spent on insecticides and half of this is used to protect cotton (Manjunath, 2004). *Bacillus thuringiensis* (*Bt*) is a ubiquitous soil bacterium first isolated

by the Japanese microbiologist Ishiwata. Some of the *Bt* strains (i.e., those that produce Cry proteins) were found to be highly toxic to insects and some plant pests. Researchers identified that Cry protein crystals bound to the receptor proteins in insect guts resulting in their death. Conversely, the protein does not affect the organism as it does not express the receptor for that particular gene product. The first commercial preparation of *Bt* was sold as a spray formulation for the control of corn pests in France in 1938.

Advanced research in gene transformation technology has made it possible to incorporate *Cry* genes into the plant cells that can target insect's larvae, which obtain food from the plant and damage it.

2.2 Rice

Vitamin A deficiency is currently affecting around 250 million people globally and every year half of million deaths are linked to this deficiency. β -Carotene (found in carrots) is the precursor for vitamin A production in the body. In addition to vitamin A, several other products in the human diet are also able to provide complex nutrients and vitamins. Only a healthy diet can save ~ 2 million children from death, which could be possible with increased prosperity; however, this is a long way off. In countries, such as India and China people survive on one bowl of rice a day; if this rice were to become more nutritious it could dramatically improve lives.

The creation of β -carotene-containing rice species proved difficult as rice does not endogenously produce the daffodil enzyme, which converts molecules into β -carotene. Researcher introduced several genes into rice to try to produce multiple products but they were unsuccessful in all instances. They then used a gene gun to produce *Agrobacterium* transgenically expressing two genes and another *Agrobacterium* expressing a different gene. The two mutant bacteria were allowed to infect the rice plants and the method was shown to be successful after all three genes were identified in the rice plants. The reason for the success of this gene transfer was the kernels' golden glow. Golden Rice is now more nutritious and one bowl of rice can provide the recommended daily amount of vitamin A to an individual. The success of Golden Rice has spurred scientists to attempt to produce transgenic white rice with higher iron content. Iron deficiency is common in mothers in developing countries and as a result premature births and low baby weights are a regular occurrence. Solutions, such as crossbreeding and the introduction of proteins that help in the absorption and storage of iron in the human body have been suggested. These rice plants could be donated to farmers to enable large-scale cultivation and dissemination of improved rice varieties to the poor.

2.3 Brinjal

Bt brinjal is GM plant containing the *Bt* gene from soil bacterium. The gene product (Cry1Ac protein) is toxic to pests that damage the fruit and shoots of brinjal. This plant was produced

by the Indian subsidiary of the US-based company, Monsanto. *Bt* brinjal was approved for human consumption and commercial use by the Indian Genetic Engineering Approval Committee on October 15, 2009, after reviewing a report submitted by Maharashtra Hybrid Seeds Company Ltd. (Mahyco).

2.4 Milk and Dairy Products

Milk has been modified with transgenes for pharmaceutical purposes without any harm to transgenic animals in most cases. Human antitrypsin, plasminogen activator, and human protein C have been introduced into the milk of sheep, goats, and pigs simultaneously. Transgenic milk has a wide range of applications in the food and pharmaceutical industries including:

1. as a food,
2. as a milk product in raw powder form,
3. as a nutrition food for infants, and
4. as a biologically active substance for use in the pharmaceutical industry.

Some oligosaccharides (i.e., lactoglobulin and casein) are very rare in human milk, but can be produced from transgenic animals that express their genes. Providing milk with an enhanced nutritive value can help to protect infants from death. Bovine growth hormones are also being used to produce pig meat with reduced levels and enhanced composition of fatty acids.

2.5 Rapeseed

Rapeseed cultivars “00” have been engineered so that they are resistant to certain pesticides and free from erucic acid and glucosinolates. Glucosinolates are toxic and thus not suitable for use in animal feed. Rapeseed has been extensively used for the production of emulsifiers and vitamin E, and refined rapeseed oil is used in the production of margarine. The high concentration of monounsaturated fatty acids (60%–70%) means that rapeseed is viewed as healthy cooking oil.

2.6 Soybean

Soybeans have been GM for herbicide resistance. GM soybeans are used in the manufacture of soy beverages, soy oil, tofu, lecithin, soy flour, pastries, breads, snack foods, baked products, fried products, edible oil products, and other specialty foods. Pioneer Hi-Bred International is the largest producer of agricultural hybrid seeds in the United States. This company created a GM soybean with a high content of the monounsaturated omega fatty acid, oleic acid (found naturally in olive oil). The monounsaturated nature of oleic acid helps in cholesterol reduction.

2.7 Maize

Maize is the only GM crop grown commercially in the European Union and it has been modified to be resistant to various pesticides. Maize is used for feeding livestock and is an important raw material in the starch industry. It is also used in the production of oil, flour, snack foods, confectionary products, soft drinks, and syrups.

2.8 Honey

Honey can also be produced from GM crops. Certain Canadian honey is obtained from bees that collect nectar from GM canola plants.

2.9 Papaya

GM papaya trees have been grown in Hawaii since 1999. These papayas are resistant to ringspot virus and show delayed maturity, which gives the suppliers more time to ship the fruits to market.

2.10 Other Genetically Modified Foods

Alfalfa contains a gene that makes it resistant to the common herbicide RoundUp, thus allowing farmers to spray the chemicals without damaging the plants. Deactivation of the polyphenol oxidase gene in apples has produced fruits that do not turn brown for 2 weeks after slicing (Nosowitz, 2015). Chicory is a popular salad green in countries, such as France and Belgium and GM has been used to introduce a gene to make the males sterile. Yellow crookneck squash has also been modified for insect resistance. This plant is very similar to zucchini and both have also been modified for virus resistance. Sugarbeets have also been modified to make them herbicide tolerant. Herbicide-tolerant flax was introduced in 2001 but was soon taken off the market when European importers refused to buy it (Young, 2013). A new variety of GM tomato has been developed that is able to produce large quantities of health-boosting compounds, such as flavanols and anthocyanins (i.e., resveratrol and genistein) (Zhang et al., 2015).

3 Production of GM Foods

Modification of the genetic complement of plants, animals, and microbes with the objective of producing desired food traits began ~1000 years ago. Such methods of modification along with natural evolutionary changes have led to the development of common food species that are now genetically distinct from their ancestors.

For a GM food to be considered as a new variety it must be genetically distinct from all other varieties, genetically stable, and genetically uniform throughout the population (UPOV, 2002). Traditionally, it took 10 years to develop, evaluate, and release a new crop

variety in accordance with international requirements. The advancements in GM methods means that it is now possible for new foods to be commercialized in a comparatively shorter time. In addition, there is also greater assurance that deleterious features of the food will be identified and harmful new varieties will be eliminated before commercial release.

3.1 Modification of Plants

Genetic modification of plants can be achieved through genetic and nongenetic engineering methods. The methods that do not employ genetic engineering include simple selection, crossing, interspecies crossing, somatic hybridization, embryo rescue, mutation breeding, somaclonal variation, and cell selection. Genetic engineering methods for the modification of plants include microprojectile bombardment, electroporation, microbial vectors, microinjection, and transposon-mediated recombination.

3.1.1 Simple selection

Simple selection is the easiest method for genetically modifying plants. A genetically heterogenous population of plants is inspected and only plants with the most desired traits (i.e., improved yield and palatability) are selected for continued propagation. New generations of plants are produced by sowing the seeds from the superior plants and all (or most) of the plants in this new generation will carry and express the desired traits. Over a period of several years the seeds of these plants are saved and replanted to increase the population of superior plants; consequently a shift in the population from inferior to superior genotype will occur.

Modern methods of simple selection, such as marker assisted selection (MAS) are being increasingly adopted. MAS is the application of molecular techniques (i.e., molecular markers, linkage mapping, and genomics) to change and improve the traits of plants based on the results of genotypic assays (Ribaut et al., 2010). In other words, this method develops improved varieties using a breeding approach based on plant genotype rather than phenotype. Genetic markers used in MAS can be defined as the biological features that are determined by allelic forms of genes and are transferable from one generation to another. Thus they can be used as probes or tags to track an individual, a chromosome, or a gene. Genetic markers can be classified into two groups: classical markers or DNA markers (Xu, 2010). Classical markers include morphological, biochemical, and cytological markers, while DNA markers are based on various polymorphism-detecting techniques, such as RFLP, RAPD, AFLP, SSR, and SNP. An overview of all genetic markers is presented in Fig. 7.1.

3.1.2 Crossing

Plant breeders use this method to combine useful features of two plants. Crossing is performed by brushing pollens from one plant onto the pistil of a sexually compatible plant. This results in a hybrid that carries genes from both parents, and the hybrid progeny can also be used as parent once it reaches flowering maturity. However, the random nature of

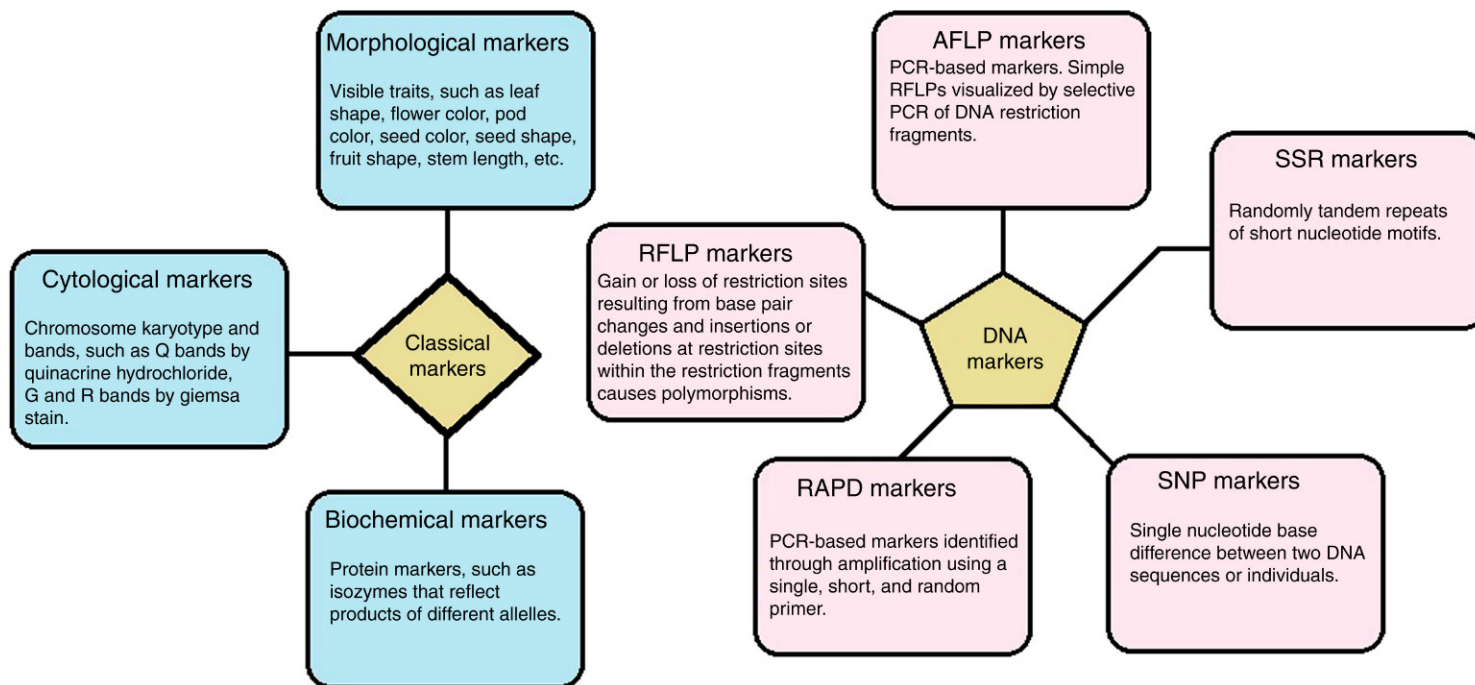


Figure 7.1: Types of Genetic Markers.

gene recombination in crossed plants means that breeders must produce thousands of hybrid progeny before they identify the plants with the maximum number of desirable traits.

3.1.3 Chromosome engineering

This is a nonrecombinant method for introducing cytogenetic modifications into DNA (Chan, 2010). Portions of chromosomes from near or distant species are recombined through a natural process known as chromosomal translocation. Various crops have been improved using this method including soybeans, rice, barley, potato, and corn (Lukaszewski, 2004).

3.1.4 Somatic hybridization

This method is also referred to as cell fusion. Cells growing in a culture medium are treated with pectinase, cellulose, and hemicellulase enzymes to remove their protective covering. These stripped cells (protoplasts) from various sources are pooled together and fused using electrical shocks. The fusion of two protoplasts produces a somatic hybrid containing genetic material from both plant sources. If the somatic hybrid is healthy and compatible it will grow a new cell wall, begin mitotic division, and grow into a hybrid plant (Rezazadeh et al., 2011). While it is easy to accomplish protoplast fusion, relatively few somatic hybrids are capable of sexual reproduction. Thus this method is not common in plant breeding.

3.1.5 Embryo rescue

Plants sometimes cross-pollinate and the resulting fertilized hybrid embryo can develop but will be unable to mature and sprout. However, with technical intervention the hybrid plant embryo can be removed before it stops growing, placed in a tissue-culture environment, and allowed to complete its development (Dayarani et al., 2014). This method is used as an intermediary step when transferring genes from distant, sexually-incompatible relatives through intermediate, partially-compatible relatives of the donor and recipient species.

3.1.6 Somaclonal variations

Somaclonal variations are the spontaneous mutations that arise when plant cells are grown in vitro. The term somaclonal variation was first used by Larkin and Scowcraft in 1981. This method has been used to grow different variants of plants in vitro (Sun et al., 2013); for example, new varieties of flax have been successfully developed and commercialized using this method.

3.1.7 Mutation breeding: induced chemical and X-ray mutagenesis

Mutation breeding aims to induce random DNA changes by exposing plants or seeds to chemical mutagens (i.e., ethyl methanesulfonate) or other mutagenic agents (i.e., ionizing radiation). The dose of the mutagen is adjusted so that the mutations are not lethal to the plants. In this method, large numbers of plants are mutagenized, grown to reproductive maturity, and multiple progeny are derived. These progeny are assessed for phenotypic expression of valuable new traits (Shin et al., 2011). The mutagenic effects are random and can occur anywhere in

the genome; for example, even if a functional mutation occurs in a particular plant it is highly likely that a deleterious mutation will also occur. Crop varieties with induced mutations are not regulated for food or environmental safety in countries, such as the United States and plant breeders do not conduct molecular genetic analysis to identify and characterize the mutations. Therefore it is highly likely that mutations other than those causing the useful traits also occur. As these are not obvious they will remain uncharacterized and their effects will be unknown.

3.1.8 Microprojectile bombardment

Microprojectile bombardment is a method for delivering naked DNA to plant cells. Microscopic pellets, with DNA attached to the surface, are shot at plant cells (Singh et al., 2010) and this method has been very effective in the modification of corn, rice, and other cereal grains.

3.1.9 Electroporation

Electroporation exploits the ability of plant protoplasts to take up macromolecules from their surroundings in the presence of an electrical impulse. Cells growing in a culture medium are stripped of their protective walls to produce protoplasts. DNA of interest is then provided to the culture medium and an electrical impulse is temporarily applied to destabilize the cell membrane and allow the DNA to enter the cells. The transformed cells have the capacity to regenerate their cell walls and develop into transgenic plants (Joannes et al., 2015).

3.1.10 Microinjection

Microinjection is a labor-intensive method that involves direct injection of DNA into anchored cells. Some of these cells survive and integrate the injected DNA.

3.1.11 Microbial vectors

Agrobacterium tumefaciens is a naturally occurring soil microbe that causes crown gall disease in susceptible plants. It transfers a portion of its own DNA into the plant cell, which becomes stably integrated in the plant genome and expressed. In the early 1980s, strains of *Agrobacterium* were developed that lacked the disease-causing genes but maintained the ability to attach to susceptible plant cells and transfer desired DNA that could stably integrate into the cells of target species. If the transformed cell regenerates into a whole fertile plant, then all cells in the progeny will carry and may express the inserted genes (Hensel et al., 2009). Most of the genetically-engineered crops that are commercially available have been produced through *Agrobacterium*-mediated transfer.

3.1.12 Transposons-mediated recombination

Transposons are pieces of DNA that are able to move from one location to another in the genome. Transposons have been extensively investigated in research laboratories, especially for the study of mutagenesis and DNA recombination (Muñoz-López and García-Pérez, 2010). However, transposons have not been harnessed to deliver novel genetic information to improve commercial crops.

3.2 Modification of Animals

Modern livestock breeds are markedly different to their ancestors due to the breeding strategies that have been developed over the past 20 years. Established and emerging technologies in animal agriculture include assisted reproductive technologies, the utilization of naturally occurring hormones (i.e., recombinant bovine somatotrophin), techniques to enhance reproductive efficiency without affecting the genome, and techniques to increase the expression of desirable genes.

3.2.1 Assisted reproductive technologies

Assisted reproductive procedures use frozen semen for artificial insemination, sire testing, and sire selection. This method has markedly enhanced the genetic quality of livestock, especially dairy cattle.

3.2.2 In vitro maturation and fertilization of oocytes

This method can produce several thousand embryos by recovering and maturing immature oocytes in ~1 day using hormone-containing media. Oocytes are then fertilized using live sperm or by injecting a single sperm into their outer layers. The resulting zygotes are cultured in vitro until the blastocyst stage before being transferred to recipient females.

3.2.3 Embryo recovery and embryo splitting

Embryo recovery and transfer allow valuable animals to contribute more offspring to the gene pool. New technologies are being developed to control the gender of the offspring and the production of single-sex sperm by cell sorting X and Y sperm will greatly benefit the livestock industry ([Campbell, 2014](#)).

Bisecting or splitting embryos yields zygotic twins (or nongenetically engineered clones) that are genetically identical in both their nuclear and mitochondrial genes. These embryos are then placed in an empty zona pellucida and transferred to recipient females who carry them to term. Maternal twins exhibit greater phenotypic variation than paternal twins (with only one X chromosome). Furthermore, differences in the mitochondrial DNA also affect the phenotype. Embryo splitting has been successfully established in cattle and other livestock and this method has become, and will continue to remain, an important factor for the multiplication of healthy offspring ([Illmensee and Levanduski, 2010](#)).

3.2.4 Transfection

Most methods for transfecting or introducing novel genes into animals are the same as those used in plants. Commonly used methods include microinjection of DNA into the nucleus of anchored cells, electroporation, lipofection, polycationic neutralization of the cell membrane, DNA introduction to improve passive uptake, and sperm-mediated transfection (which is usually performed in conjunction with intracytoplasmic sperm injection).

3.2.5 Retroviral vectors

Retroviral vectors are proficient transfer systems for introducing foreign genes into target cells. The prevalence of retroviral-based vectors is partly due to their long research history, which paved the way for the successful manipulation of the retroviral genome (Yi et al., 2011). Retroviral vectors are derived from RNA viruses, which are capable of reverse transcribing their genome into double-stranded viral DNA to enable stable insertion in the host genome (Fig. 7.2). RNA viruses that are extensively used for virus vector engineering include murine leukemia viruses and lentiviruses (Walter and Stein, 2000).

3.2.6 Transposons

The use of transposable elements in animal cells has not been completely developed. No active naturally-occurring transposable elements have been found in mammals but transposable elements from insects and fish are under investigation.

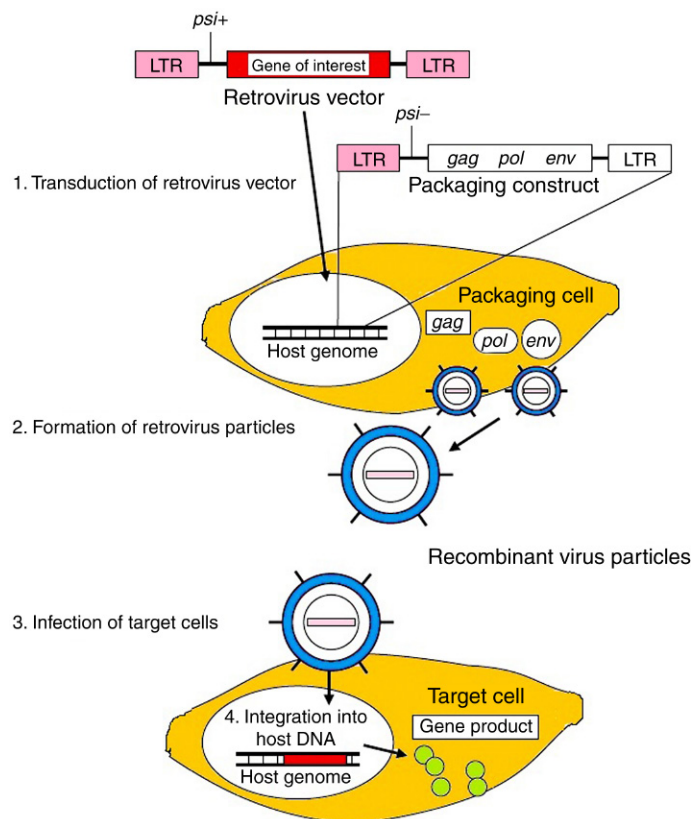


Figure 7.2: Principles of Retroviral Vector Gene Transfer.

3.2.7 Knockin and knockout technology

Transgenic technology has been used to create organisms that lack specific genes or have a specific gene replaced by another (Nakashima and Miyazaki, 2014). The addition (knockin) or deletion (knockout) of specific gene functions through introduced mutations or homologous recombination has become a common technology for manipulation of the animal genome (Bernheim et al., 2016).

4 Risks Related to Genetically Modified Foods

New combinations of genes that result from the genetic manipulation of existing genes using conventional breeding techniques may introduce several unintended and unexpected effects. However, the selection process does eliminate genetic lines that express undesirable traits. Plants and animals modified through conventional breeding methodologies are consistently assessed for changes in productivity, their reaction to diseases, reproductive competency, and quality characteristics. These organisms are not evaluated for unintended effects at the molecular level and are rarely subjected to toxicological safety assessments. The application of genetic modification methods and the potential for unintended adverse effects are similar for both plants and animals. Thus, information obtained from plants can also be used to identify concerns related to the genetic modifications of animals. Certain crops naturally produce allergens, toxins, or other antinutritional substances. Plant breeders and agronomists monitor levels of the hazardous antinutritional substances and if a particular breeding line produces high amount of toxins it is eliminated from commercial consideration.

The use of gene technology in food production has produced ethical dilemmas concerning the negative effects of GM foods. The concerns related to GM foods are classified as environmental hazards, human health risks, and economic concerns.

4.1 Environmental Hazards

GM crops may cause unintended harm to other organisms. For example, pollen from *Bt* corn causes high mortality rates in monarch butterfly caterpillars that consume milkweed plants. The fear is that if pollen from *Bt* corn is blown onto milkweed plants in neighboring fields, the caterpillars could eat the pollen and perish due to the presence of *Bt* toxins. Another risk is the development of highly resistant organisms. GM crops have led to a reduction in the effectiveness of pesticides as some mosquito populations have developed resistance to the now banned pesticide DDT. In addition, the transfer of genes to nontarget species is of concern; for example, the transfer of herbicide resistance genes from GM crops into weeds may result in the creation of “superweeds” (Verma et al., 2011).

GMOs may also pose a series threat to biodiversity. When an exogenous gene is inserted into a receptive organism the gene network is perturbed. This perturbation alters the orchestration of the network resulting in the breakdown of epistatic relations and provokes alterations in feedback mechanisms that regulate gene expression (Hug, 2008). One example of the adaptation to a new gene is seen in GM soybeans. The insertion of *CPY-EPSPS* from *Agrobacterium* disturbed the soybean gene network and resulted in altered metabolism and lignin production. As such, the stems of the plants were unable to withstand heat and they broke down. The integration of a foreign gene may be more disadvantageous than advantageous as it modifies a biological model that was the result of a long evolutionary process. The effect of genetic modification is an increase in the plant's genetic load and this genetic load is inversely related to the adaptive value. Thus GMOs tend to possess less adaptability. The introduction of GM crops into native ecosystems may also result in the flow of DNA from GM crops to wild relatives, which may affect the genetic identity, as well as the integrity of wild populations and the local genetic diversity.

4.2 Human Health Risks

Potential health risks associated with the use of GM foods include unexpected gene interactions, risk of cancer, allergies, and horizontal gene transfer (HGT). Certain gene constructs interact unexpectedly to produce harmful effects; for example, some feeding studies have shown that GM diets have a minor effect on animal weight. It is believed that these unexpected results are due to the inserted gene or the side effects of the genetic transformation, which can seriously disturb metabolism. GM crops possess more pesticide residues than non-GM crops. The main component of some pesticides is glyphosate, which has been linked to the development of non-Hodgkin lymphoma. GM foods may also cause allergenicity due to the presence of new proteins or through the interaction of new proteins with endogenous proteins. The assessment of the allergenic potential of novel foods presents a serious problem as no reliable tests are available for predicting allergenicity.

HGT is the transfer of genetic material to a member of the same species, a different species, or a different domain of life. It has been suggested that ~20% of GMOs with engineered genes from viral pathogens may generate new viral strains with unknown properties. HGT plays a major role in the adaptation of bacteria to new environments compared to the alteration of gene function through mutations (Hug, 2008). HGT may also lead to the transfer of antibiotic resistance genes from GM plants to human gut bacteria (due to the transformation of bacteria in the food chain), soil bacteria, or plant-related microbes.

4.3 Economic Concerns

It is important to note that genetic engineering policies might not be favorable for developing countries. First, many innovations will remain unattainable for most citizens of developing

countries. Second, GM crops may lead to the extinction of native cultures in developing countries and prove to be detrimental to agricultural workers; for example, the use of herbicide-resistant GM crops reduces the labor demand. Third, focus on GM-related applications may diminish efforts to explore other ways of enhancing agriculture (i.e., improving seed production and distribution). Finally, GM crops may pose a threat to the welfare and autonomy of farmers who wish to produce non-GM products.

5 Methods for Assessing the Safety Profiles

GM foods are novel and the assessment of their safety is essential to protect the environment, as well as the health of humans and livestock. It is also necessary to tackle the risks related to the use of GMOs. To assess the nutritional quality and chemical composition of food in relation to the environmental effects, genetic alterations, and mutations (naturally occurring or induced), it is important to devise analytical procedures that determine:

1. The absolute structural identification of all compounds in a sample.
 2. The absolute quantification of all compounds in a sample, taking into account recovery and detection sensitivities.
 3. The biological impact of each compound alone and in a complex mixture at a given dosage.
 4. Changes to the metabolism and physiology of a target food organism as a result of the transgenic events, and the biological consequences of these changes to human health.
- These should be performed through predictive modeling.

There are two basic analytical approaches for the safety assessment of GM foods: targeted quantitative analysis and simple profiling techniques.

5.1 Targeted Quantitative Analysis

Targeted quantitative analysis is a traditional approach in which a method is established to enumerate a predefined compound or class of compounds. An important goal of the analytical evaluation of GM foods is measuring the content of relevant essential micronutrients (e.g., vitamins and minerals), essential macronutrients (e.g., essential amino acids and fatty acids), antinutrients (e.g., enzyme inhibitors and lectins), nonessential nutrients of importance to health (e.g., dietary fibers), and known toxins. Primary analytical attention should be directed toward the compounds of utmost importance; for example, legumes, such as soybeans do not contain a significant source of dietary ascorbic acid, but they can be an important source of folate. Thus analytical requirements and priorities should be established accordingly.

It is also crucial to consider the nature of the introduced genetic changes when establishing the analytical requirements. For example, if the genetic change has altered the total protein content, the expression of a specific protein, or the synthesis of one or more amino acids, then one should analyze the full complement of amino acids during the assessment.

The concept of nutrient bioavailability is very complex; hence, it is important that factors that impact on bioavailability be well characterized to merit their inclusion in the targeted analysis of a particular food. For example, phytate is a common constituent of cereal grains and it is able to affect the bioavailability of many divalent cationic minerals (i.e., zinc and iron). To provide a full picture of the genetic and compositional changes in genetically engineered foods, targeted analysis using modern molecular biology techniques should be used. For example, sequencing upstream and downstream of the transgene will provide information on the insertion site and whether the insertion has disrupted any other genes or regulatory elements. Targeted analysis of the transgene transcript using gene-specific probes, northern blotting, or real-time PCR may also be necessary to verify and quantify the extent, developmental timing, and tissue specificity of the transgene expression. This will also determine whether other adjacent genes are impacted by the expression of the transgene. Measuring expression of the protein encoded by the transgene is also crucial for food safety assessment.

5.2 Profiling Methods

Profiling methods involve untargeted analysis in an extracted biological sample with the aim of determining the patterns of detected constituents. For proteins and metabolites, profiling is performed using chromatographic, electrophoretic, or spectral methods, while the profiling of nucleic acids involves sequence-specific hybridization methods. The major advantage of profiling methods is that they compare patterns of constituents and detect compositional differences without the requirement for identifying all of the compounds or understanding the functions of all genes in an organism. However, profiling methods are limited by issues of sensitivity, constraints of sample extraction, and preparation methods, and artifacts generated during sample handling.

One of the most commonly used metabolic profiling techniques is gas chromatography–mass spectrometry (GC–MS). Plant tissues are extracted using combinations of organic and aqueous solvents and the compounds present in each extraction are then analyzed. Absolute structural determination of the original molecules is not possible using this method as derivatization leads to the compound modifications. Despite the limitations of differing solubility and compound modification, GC–MS has been successfully used to reproducibly resolve 100–500 compounds and it has also been used for profiling *Arabidopsis thaliana* and potato tubers. An alternative to GC–MS analysis is liquid chromatography–mass spectrometry (LC–MS) analysis. Liquid chromatography is advantageous because compounds do not have to undergo derivatization and can be resolved in intact form. The LC separation may be coupled to a variety of detectors; for example, the coupling of LC to a photodiode array detector has been used for profiling isoprenoids in tomato (Fraser et al., 2000). LC–MS is more advantageous than photodiode array as more metabolic compounds can be separated and detected (Huhman and Sumner, 2002). The disadvantages of LC–MS include matrix

effects, whereby the presence of one compound in the spectrophotometer affects the ability to detect and quantify another compound.

Nuclear magnetic resonance (NMR) spectroscopy is the third approach for metabolite profiling. NMR possesses the ability to provide fingerprints with good chemical specificity for compounds that are present in comparatively high abundance in an extract. Unlike the previous methods of profiling, NMR can be performed on whole tissues and can also be employed for the analysis of extracts or fractionated extracts and interfaced with LC-methodologies. NMR can be performed relatively rapidly and with moderate to high throughput.

6 Testing and Labeling of Genetically Modified Foods

Foods derived from GMOs have already appeared in the European Union (EU), Japan, Australia, and Malaysia. It is essential to identify these foods and label them appropriately so that consumers can choose whether they want to consume GM, conventional, or organic foods. However, the labeling of GM foods is not mandatory in the United States.

The EU introduced a novel food classification system in 1997 where novel foods were categorized as: (1) foods that are GMOs or that contain GMOs (e.g., yoghurt and tomato); (2) foods that are produced from GMOs (e.g., oil extracted from herbicide resistant soya, enzymes, and vitamins).

GMO contamination of 0.9% has been set as the threshold for labeling GM foods. All current and future products should be irreprehensible to health, the environment, society, and ethics. In the latest EU legislation (EU No. 1829/2003 and 1830/2003) GM food is removed from the novel food classification. For the purpose of labeling it is important to be able to identify foods that are GM.

Testing for GMOs in food and feed is routinely performed using sophisticated molecular techniques, such as DNA microarray and quantitative polymerase chain reaction (qPCR). A summary of the technologies and the targets used in the analysis of GMOs is provided in [Table 7.2](#). The basis of these tests involves the screening of genetic elements (i.e., p35S, pat, tNOS, or bar) or event specific markers for the official GMOs (i.e., Bt11, Mon810, or GT73) ([Bawa and Anilakumar, 2013](#)). The DNA microarray-based method uses array technology to screen samples for different GMOs, while the qPCR method uses specific primers to screen elements or events specific markers. Controls are necessary to avoid false-positive or false-negative results; for example, a test of cauliflower mosaic virus (CaMV) is used to avoid a false positive in the event of a virus-contaminated sample. The general methodology of testing for GMOs using the PCR-based detection system is depicted in [Fig. 7.3](#).

PCR-based methods suffer from certain limitations, such as minimum DNA requirements, which are affected by food processing techniques. Thus DNA that cannot be amplified will affect all qPCR detection systems. To navigate these limitations, [La Mura et al. \(2011\)](#)

Table 7.2: Techniques and targets for the detection of GMOs (Fraiture et al., 2015a).

Technology	Multiplexing	Methods	Targets
ELISA	Single	—	CP4-EPSPS, Cry1Ab, Cry1Ac, pat, GOX, and CpTI
Immuno-PCR	Single	—	Cry1Ac, p35S, and tNOS
qPCR	Single	Plant specific	RBCL, LEC, ADH, and PLD
		Element specific	P35S, tNOS, pat, t35S, bar, and pFMV
		Construct specific	Gat-tpinII
		Virus specific	CRT
		GM specific	DAS-40278, MIR162, MIR604, MON810, MON863, and NK603
	Duplex	Element specific	P35S, tNOS, bar, and pat
	Triplex	Taxon specific	LEC and Zein
	Pentaplex	Element specific	p35S, tNOS, bar, pat, CTP2/CP4-EPSPS, and LEC
PCR capillary gel electrophoresis method	Hexaplex	Construct specific	SAMS and LY
	Tetraplex	Taxon specific	Zein, LEC, and SAD1
		Element specific	p35S, tNOS, Cry1Ac, p35S, and tNOS
	Pentaplex	Event specific	Bt11, GA21, NK603, and MON810
		Taxon specific	ADH
	Hexaplex	Event specific	DAS-59122, LY038, MON88017, MIR604, and 3272
PCR microarray	Octaplex	Event specific	TC1507, MON531, NK603, and GA21
	Nonaplex	Event specific	Bar, chy, pAct, CP4-EPSPS, and Cry1Ab
	Duplex	Element specific	p35S and tNOS
	Triplex	Element specific	Pat, Cry1A, and CP4-EPSPS
	Tetraplex	Taxon specific	IVR, LEC, and CRU
	Octaplex	Event specific	Bt176, Bt11, and MON810
	Decaplex	Event specific	MON1445, Bt176, and GTS40-3-2
	Dodecaplex	Event specific	Bt 176, Bt11, MON810, GA21, CBH351, and DBT418
Luminex technology	Simplex	Element specific	p35S and CP4-EPSPS
	Triplex	Event specific	MIR604 and MON88017
	Tetraplex	Event specific	Bt176, MON810, NK603, and GA21
	Pentaplex	Element specific	Cry1Ac, tNOS, p35S, and LS28
	Hexaplex	Element specific	Cry1Ac, Cry1F, and pat
Digital PCR technology	Simplex	Taxon specific	HMG, LEC, GLU, and CRU
	Duplex	Event specific	HMG
LAMP strategy	—	Taxon specific	ADH, LEC, PLD, and IVR
	—	Element specific	pFMV, p35S, aadA, uidA, pNOS, bar, pat, phy, Cry3A, and Cry2A
DNA walking	—	Cassette PCR	MON863, NK603, T25, Bt11, GA21, Bt176, CHB-35, GTS40-3-2, GT73, TC-19, and TT51-I
	—	LT-RADE	MON810, T45, LLRICE62, and LLCOT-TON25
	—	TAIL-PCR	MON863, KeFeng-6
	—	SiteFinding-PCR	KMD1, and MIR162

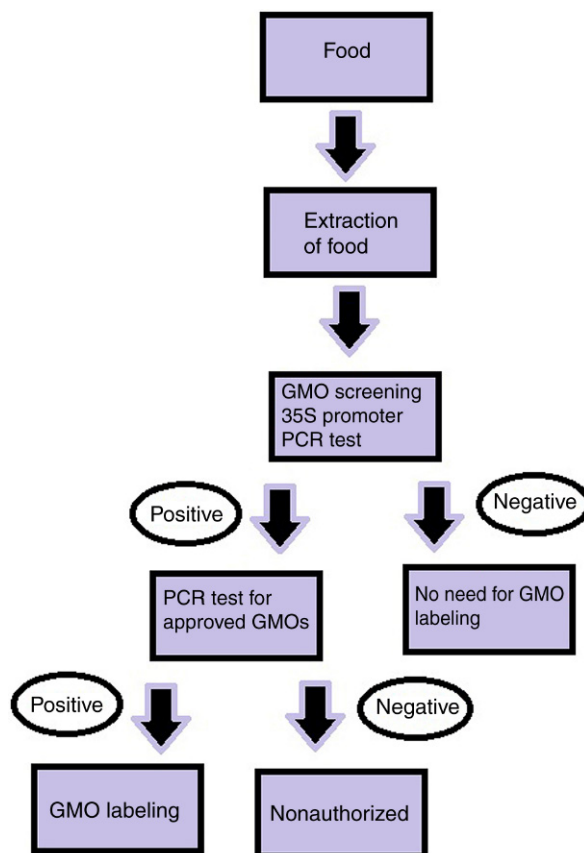


Figure 7.3: General Methodology of Testing for Genetically Modified Organisms.

applied quantization using informative zeroes (QUIZ) to estimate the contents of RoundUp Ready soya and MON810 in processed foods containing one or both GM varieties. The authors established that QUIZ methodology permits the quantification of a sample without the need for certified reference materials.

6.1 qPCR Technology for Detection of Genetically Modified Organisms

qPCR is one of the most common strategies to detect, identify, and quantify GMOs and it can be performed using SYBR Green or TaqMan chemistries. Primers that are specific for the target gene are used in both chemistries. PCR amplification is recorded in real time through the detection of fluorescence originating from the asymmetrical cyanine dye binding to double-stranded DNA (SYBR Green) or from the flurogenic probe binding to the target sequence (TaqMan). The later technology is favorable for the analysis of both unprocessed and processed food/feed matrices.

There are three main steps followed in the routine qPCR analysis of GMOs: (1) The presence of a GMO is assessed using a screening approach that targets the most common transgenic elements found in GMOs (i.e., tNOS, a nopaline synthase terminator from *A. tumefaciens* and p35S, a 35S promoter from CaMV). More discriminative markers (i.e., gat-tpin-II, Cry3Bb, and t35S pCambia) and certain taxon-specific markers can also be used. This step allows the user to establish a list of GMOs that may be present in the tested samples and thus prevent further unnecessary assays. Most of these markers are validated based on minimum performance requirements at the EU level and are included in the compendium of reference methods for GMO analysis (Fraiture et al., 2015a). (2) Potential genetic modification events are detected in this step using construct-specific markers (e.g., markers that target the junction between two elements inside the transgenic cassette) or event-specific markers (e.g., markers that target the junction between the transgenic cassette and the plant genome). Event-specific markers are currently favored for the appropriate discrimination of each genetic modification as they target the unique transgenic integration sites. (3) The number of genetic modification events present in the tested food/feed sample is determined using event-specific and taxon-specific markers, which will allow the user to identify the number of copies belonging to the transgene and to the endogenous genome. A real-time, PCR-based, ready-to-use, multitarget, analytical system has been developed to allow the simultaneous identification of 39 genetic modification events. This system contains a combination of several taxon-specific, event-specific, and construct-specific TaqMan markers in a 96-well prespotted plate (Querci et al., 2010).

A number of rapid and cost-effective systems have been developed to facilitate the interpretation of qPCR results. The combinatory SYBR Green qPCR screening platform has been developed, to support decisions at the screening level. It combines the experimental C_t and T_m values with the 20 SYBR Green methods running in a single 96-well plate, and targets plant genes, taxon genes, and transgenic elements (Broeders et al., 2012; Fraiture et al., 2014). Reliable information on GMOs can be obtained from GMO-seek and GMO-finder databases. After the interpretation of the experimental results (gained from the in-house or EU reference methods) the names of the positive elements are introduced into the databases so as to provide a list of potential GMOs that will need to be experimentally verified (Block et al., 2013). The JRC-GMO-Matrix platform combines information from the GMOMETHODS database and the Central Core DNA Sequences Information System. This platform amalgamates all positive and negative signals experimentally observed within the EU with the aim of increasing the reliability of predicting potential amplified genetic modification events. The JRC-GMO-Amplicons, which contains publicly available putative GMO-related sequences, also strengthens the JRC-GMO-Matrix platform (Angers-Loustau et al., 2014).

The qPCR method is highly flexible, simple, rapid, and sensitive but its success depends on a number of factors; for example, the throughput of the qPCR strategy is usually limited to one marker per reaction. Additional markers need to be developed and used to fully permit

the detection of the increasing number of GMOs; however, this is likely to make the analysis complex and laborious. qPCR technology also only targets known sequences, therefore a negative signal assures the absence of the known GMOs but unknown GMOs may still be present. Finally, inhibitors, such as polyphenols, polysaccharides, pectin, xylan, and lipids can interfere with the efficiency of the PCR reaction and result in an underestimation or a masking of the amount of GMO present in a sample (Demeke and Jenkins, 2010; Opel et al., 2010; Schrader et al., 2012).

6.1.1 Multiplex qPCR strategy

Multiplex qPCR can be used to detect several DNA targets in a single reaction. The major advantage of this technology is that it decreases the number of reactions necessary to evaluate the presence of GMOs. A number of multiplex qPCR TaqMan strategies have been developed, which mainly involve screening markers, such as p35S and tNOS (Cottenet et al., 2013). The development of optimum procedures for multiplex assays is highly challenging with regards to primer and probe design. The throughput of this methodology is also limited by the availability of dyes that possess fluorescence emission and absorption spectrum that are sufficiently distinct to avoid overlapping signals. The combination of different dyes also increases the risk of elevated background fluorescence and thus the majority of reported multiplex PCR assays have only amplified two or three targets simultaneously. A maximum of six markers have been successfully detected in one assay (Bahrdt et al., 2010).

6.2 PCR Capillary Gel Electrophoresis Technology

PCR capillary gel electrophoresis uses fluorescently labeled primers that allow different amplicons of the same size to be distinguished. The resolution power of a capillary gel electrophoresis system is much better than that of simple electrophoresis gel; however, the sensitivity of this technology is comparatively lower than that of qPCR (Milavec et al., 2014).

6.3 Microarray Technology for the Detection of Genetically Modified Organisms

GM targets are first amplified by PCR using target-specific and/or universal primers before being hybridized on the array. The arrays permit the simultaneous detection of more than 250,000 targets in one assay (Nakaya et al., 2007) although it is slightly less sensitive than qPCR. Multiplex quantitative DNA array-based PCR is one method for testing the presence of GMOs in food/feed samples. An initial PCR is conducted using target-specific primers that harbor a universal tail (which allows the use of universal primers in the second PCR). The signal is identified after hybridizing PCR products with fluorescently labeled probes on the DNA array. This approach has been tested on transgenic maize (Rudi et al., 2003).

Padlock probe ligation-microarray detection systems have also been used to detect GMOs in maize, cotton, and soybean. In this system the targets are initially hybridized to linear

padlock probes harboring target-specific and universal sequences to be amplified before these sequences are amplified using universal primers (Prins et al., 2008).

6.4 Luminex Technology

Luminex technology is used to analyze biotinylated targets amplified by single or multiplex PCR assays. This technology is efficient at detecting up to 500 different targets in a single sample using spectrally distinct beads that are independently coupled to distinct nucleic acid probes. Once biotinylated oligonucleotides are hybridized to the corresponding probe-bead complexes, the reader device can individually analyze each microsphere using flow cytometry. Laser excitation of 635 and 532 nm permits the identification of bead sets and determines the presence or absence of the target, respectively (Fraiture et al., 2015a). The liquid bead array is more sensitive and faster than the microarray system, hence, the Luminex Technology appears to be a promising alternative for the detection of GMOs. The only shortcoming of this technology is the limited number of studies using this technology, thus further experiments are required to provide effective and validated systems.

6.5 Digital PCR Technology

Digital PCR (dPCR) technology has been successful in resolving the difficulties observed during the relative quantification step in qPCR due to the presence of PCR inhibitors or low copy numbers of GMOs. This technology is based on the binomial Poisson statistics, so that each partition of the fractionated sample is determined as positive or negative by the dPCR. This allows absolute quantification of the number of nucleic acid targets from GMOs.

Chamber dPCR and droplet dPCR have been developed for the detection of GMOs. In chamber dPCR the sample is partitioned into several thousand microfluidic chambers. GM maize MON810 has been targeted with this approach using a duplex PCR composed of the MON810 event-specific and maize taxon-specific methods (Burns et al., 2010; Corbisier et al., 2010). In droplet dPCR several thousand droplets are generated by water-oil emulsion and a simplex (or duplex) PCR with the MON810 event-specific and maize taxon-specific methods can be performed (Morisset et al., 2013). dPCR technology has the potential to be used for the detection of GMOs as it is absolute and not relative. The measurement does not necessarily require the use of a reference material and the PCR efficiency is less impacted by the presence of inhibitors.

6.6 Loop-Mediated Isothermal Amplification (LAMP)

LAMP uses four primers that are specific for six distinct regions of the target. It allows initiation of the reaction and enhances the amplification speed by forming a loop structure under isothermal conditions. Amplification can be visualized in the tubes due to presence of fluorescent dyes. Several LAMP markers have been developed to target transgenic elements

(Cheng et al., 2014). LAMP can tolerate the presence of several PCR inhibitors (i.e., acidic polysaccharides) and the implementation of this method does not require any sophisticated devices. The reaction can even be carried out in a water bath or a simple heating block (Xu et al., 2013). The limitation of this method is the difficulty in designing the four primers per target, which is required to guarantee the high specificity and sensitivity.

6.7 DNA Walking

DNA walking permits the identification of unknown nucleotide sequences adjacent to already known DNA regions in any given genome. It uses primers that are specific for the known sequence and primers that are dictated by the DNA walking method of choice. The final product is then sequenced using Sanger technology and analyzed against available databases. Depending on the characteristics of the first step, DNA walking methods can be categorized into three groups: (1) Restriction-based methods, whereby DNA digestion is performed using appropriate restriction enzyme sites close to the sequence of interest (i.e., the junction between known and unknown sequences). The restriction fragments obtained are then either selfcircularized or ligated to DNA cassettes (Leoni et al., 2011). Many transgene flanking regions and unnatural associations in GM tobacco, potato, and *A. thaliana* have been characterized using this method; (2) Extension-based methods, which are characterized by extension of a sequence-specific primer. The resulting single-stranded DNA is subsequently ligated to either a DNA cassette or a 3'-tail. This technology has been successfully applied in GM maize, rice, soybean, and cotton; (3) Primer-based methods, which combine combinatorial primers to target-specific primers designed on the basis of various PCR strategies.

Since DNA walking methods do not require prior knowledge of the sequence of interest, GMOs with entirely or partially known sequences can be characterized. DNA walking methods can also be easily integrated into the routine analysis of GMOs as similar primers are used for qPCR analysis and DNA walking methods. Therefore DNA walking is a simple and rapid approach that could be easily incorporated by laboratories to verify signals previously obtained in qPCR without additional costs or equipment requirements.

DNA walking methods are less sensitive and specific than qPCR. Some of these methods also require laborious, complex, and highly time-consuming techniques. For this reason, DNA walking approaches have been developed, which involve two seminested PCR rounds so as to increase the yield and specificity of the GM targets (Fraiture et al., 2015b).

7 Conclusions

Biotechnology has been used to improve the quality and yield of foods in many parts of the world. It is highly specific and relatively rapid when compared to traditional breeding techniques. A comprehensive safety assessment is conducted on GM foods before the

products are commercialized, which includes the evaluation of proteins used in these foods for their potential allergenicity and toxicity, as well as compositional analysis of the food. Studies that perform this assessment have required significant resources, equipment, and training and they have collectively demonstrated that agricultural biotechnology is not inherently dangerous or harmful. The safety assessment standards for GM foods are comprehensive and have proven to be extremely effective. Countries where GM crops have been developed have benefited from agricultural biotechnology and developing countries with emerging economies will also benefit from the application of this technology.

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Awareness and Utilization of Genetically Modified Foods in Nigeria

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1 Introduction

Genetically modified foods (GMFs) are plant and animal products derived from plants and animals whose DNA has been altered through genetic engineering. Genetic engineering allows scientists to introduce new traits in plants and animals and have greater control over existing traits compared with conventional methods of selective and mutation breeding (GM Science Review First Report, 2003). Specific traits in plants and animals can be modified or completely exchanged with a foreign gene to ensure better performance of that animal or plant. Calgene was the first company to market GMFs. The Flavr Savr tomato, with delayed-ripening, was introduced by the company in 1994 although its commercialization was unsuccessful. Examples of other GM crops include cassava, soybean, papaya, corn, cottonseed, and oil. Some of these crops have been modified for increased disease resistance whilst others have been modified to produce increased yields in a shorter time frame. GM animals have also been developed and these are referred to as transgenic animals because they contain genes from other animals. For example, Dolly the sheep was engineered so that the gene controlling milk production was replaced with the human homologue, thus allowing Dolly to produce human milk. However, as of November 2013 no GM animals were available in the market. This could be due to the controversies surrounding genetic modifications, the cost, or the technologies involved. Despite the importance of GM plants and animals, there are controversies associated with this technology, such as food safety, labeling, regulations, and research methods. Other concerns include the potential impact on the environment and the concerns that some GM organisms are subject to intellectual property rights owned by a few big corporations (Cowan, 2011). Fig. 8.1 shows major GM crops that have been commercialized globally.

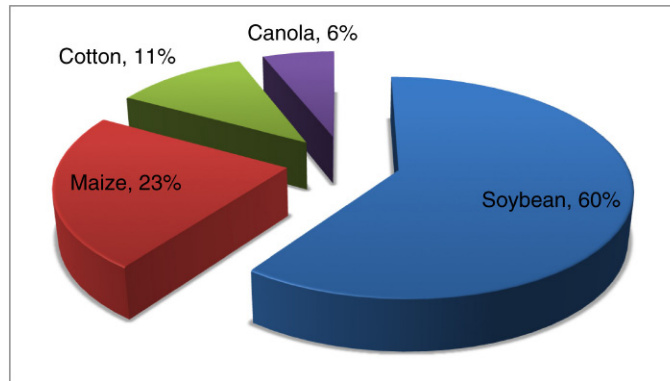


Figure 8.1: Genetically Modified Crops That Have Been Commercialized Globally. James, C., 2004. *Preview: Global Status of Commercialized Biotech/GM Crops: ISAAA Briefs No. 32, International Service for the Acquisition of Agri-Biotech Applications. Ithaca, New York.*

2 Nigeria as a Country

According to Wikipedia, Nigeria is a federal constitutional republic in Western Africa that gained independence from Britain in 1960 (<https://en.wikipedia.org/wiki/Nigeria>). In 2016 Nigeria had a population of 170 million. It shares borders with the Republic of Benin (west), Chad (east), Cameroon (east), and Niger (north). The southern part of Nigeria borders the Gulf of Guinea in the Atlantic Ocean. Nigeria contains 36 states and the Federal Capital Territory, which is located in Abuja. Nigeria is a democratic secular country. According to Wikipedia, Nigeria hosts numerous kingdoms and tribes, which are scattered over the country (<https://en.wikipedia.org/wiki/Nigeria>). The largest ethnic groups are Hausa, Igbo, and Yoruba and the official language is English. There are three major religions practiced in Nigeria: Christianity is practiced by Christians living in southern Nigeria; Islam is practiced by Muslims living in northern and western Nigeria; and traditional religion, which is practiced by a few Nigerians. Nigeria is called the “giant of Africa,” because of its size (from <https://en.wikipedia.org/wiki/Nigeria>). Nigeria had the world’s 20th largest economy in 2015 due to a GDP of more than US \$500 billion and purchasing power parity of US \$1 trillion; however, just 1 year later Nigeria was in serious economic crises due to mismanagement of funds and resources by leaders and reliance on a mono-economy (i.e., crude oil). The global recession has taken a toll in Nigeria and everyone is looking for a way out: will the genetic modification of plants and animals be the answer?

3 Agriculture in Nigeria

Agriculture used to be the main foreign exchange earner for Nigeria (Ake, 1996); however, the discovery of crude oil 40 years ago directed the attention of the Nigerian government away from agriculture and led to the downfall of this sector. In 2010 approximately 30%

of Nigerians were employed in the agricultural sector ([Labour Force Statistics 2010](#)). Some major crops found in Nigeria include palm kernels, palm oil, beans, cashew nuts, sesame, cassava, groundnuts, cocoa beans, kolanut, maize, gum arabic, melons, millet, plantains, rice, rubber, sorghum, and soybeans. These crops are not produced at full capacity today because of the failure of the government to invest in agriculture (i.e., they failed to provide soft loans for farmers, feeder roads, and electricity for the preservation of perishable food items). The neglect of the agricultural sector has meant that agriculture has failed to keep pace with the country's rapidly growing population ([Pasquini and Alexander, 2005](#)).

[Total Facts About Nigeria \(2016\)](#) stated that agricultural practice in Nigeria has increased gradually over the last few years thanks to the participation of the private sector (who understand the economic potential of agriculture) and the advent of modern technologies and their associated infrastructures. In the past, most Nigerians were involved in subsistence farming to generate food for their families. The low yield of farm produce meant that only a small amount of produce was available for sale in the markets. The Nigerian soil is extremely fertile and the climatic conditions are conducive for the cultivation of a wide variety of crops, including maize, yams, millet, beans, sorghum, onions, potatoes, rice, carrots, pears, okra, cocoa, and cocoa yam.

Wikipedia places Nigeria as the world's number one producer of cassava, and this contributes over 45% of the country's agricultural GDP (<https://en.wikipedia.org/wiki/Nigeria>). The rearing of animals and the planting of crops contribute ~20% of the GDP, but this falls behind the oil industry, which has become the major domestic produce of Nigeria ([Total Facts About Nigeria, 2016](#)).

4 Animal Rearing in Nigeria

Animal rearing is one of the major farming practices in Nigeria, especially in the northern region of the country where the savannah region provides ample grasses. These animals are used for food and transport (of goods and man) whilst others are sold during Muslims and Christian festivals. Livestock farming is an important aspect of agriculture in Nigeria and Nigerians depend on meat from cattle, sheep, goats, and chickens. Examples of animals reared in Nigeria include pigs, cows, goats, horses, and sheep whilst birds reared in Nigeria include ducks, pigeons, chickens, and turkeys ([Total Facts About Nigeria, 2016](#)). Large modern poultry farms are scattered all over the country and provide meat and eggs to eateries. However, despite the presence of poultry farms, frozen chickens and turkeys were regularly imported into the country. A ban on the import of these products has come into force and this has boosted the production of these birds in Nigeria. Whilst pigs, goats, sheep, and related animals are reared on farms, those who keep cattle move from one part of the country to another in search of grasses especially during the dry season.

5 Problems and Proffered Solutions of Agriculture in Nigeria

To overcome the challenges in the Nigerian agriculture sector the government at all levels must rise up to the task by establishing and implementing policies that will promote agriculture. There is also a need for proper empowerment, education/orientation of farmers so that they are able to use modern farming techniques. This education is especially important for the farmers who practice subsistence agriculture so that they have access to operational methods that will enable them to improve agricultural systems and increase outputs. Another major problem in Nigerian agriculture is electricity. Power is required for the storage and processing of products but problems with electricity have contributed to ~40% of Nigerian agricultural produce becoming spoilt after harvest. A constant electricity supply would mean that seasonal perishable food items (i.e., vegetables, fruits, milk, and meat products) could be stored or processed so they are available all year round. In addition, the lack of adequate road networks between farms and cities means that transporting harvested produce to markets is a serious challenge. The impact of this is seen in the high cost of farm produce, which is connected with the high cost of transportation. The high cost of transportation is a discouraging factor that prevents many farmers from producing or cultivating large quantities of plants or animal products. Finances that are essential for establishing and maintaining farm infrastructures are lacking and the challenge that this poses in Nigeria cannot be overemphasized ([Total Facts About Nigeria, 2016](#)). There are many farmers who are willing to farm if the land and resources are provided for them.

Representative Muktari Muhammad Chiromawa emphasized that the major problem of agriculture in Nigeria is predominantly related to seeds ([Abubakar, 2015](#)). Improved seed varieties are lacking in Nigeria and this has affected the annual quantity and quality of crops. He further emphasized the problem of governance, and articulated that this must be taken as a priority if agriculture is to be improved. He also stated that the Nigerian government has not done enough despite the natural resources God blessed Nigeria with (i.e., good climate, water bodies for irrigation, fertile soil, and manpower). Furthermore, he emphasized the need to restructure the Agricultural Research Council of Nigeria so that they are able to execute their mandate of monitoring, coordinating, supervising, and providing recommendations about Nigerian agriculture. If this occurs, improved policies could be implemented that would boost the production of cash and food crops, as well as livestock animals in Nigeria, thus mitigating the problem of food security in the country.

Another problem is that the results of research findings, which are capable of promoting agricultural activities in Nigeria, lie on the shelves of research institutions ([Abubakar, 2015](#)). This is a failure of Nigerian agricultural extension services and all hands must be on deck to ensure that research findings are translated to farmers who need them to enhance productivity. Most activities in the world today depend on science and technology and Nigerian agriculture must embrace these sectors to enhance productivity. This is likely to be in the form of

mechanization, as well as the use of hybrid or GM seeds. Farmers must also be trained to use these technologies and equipments. The Nigerian government could buy modern farming tools and equipment and provide them to farmers at subsidized costs. [Abubakar \(2015\)](#) recommended the establishment of a single entity that collates research findings and disseminates them to the applicable farmers. The farm extension services of the Ministry of Agriculture and the Agricultural Development Program must be encouraged to perform extension to bridge the gap between the farmers and the research institutions. For instance, studies of guinea corn in Nigeria highlighted four varieties, but unfortunately many farmers have not been made aware of these improved varieties. This disconnect could be blamed on the poor dissemination of research findings ([Abubakar, 2015](#)).

Lack of improved breeds is another major problem of livestock production in Nigeria. Emphasis must be placed on replacing indigenous breeds with breeds of cattle (and other livestock animals) that have high yielding milk abilities. Most Nigerian animals are small in size but resistant to diseases and harsh environments; however, their exotic counterparts are bigger and are able to produce more milk and eggs. These animals are able to support themselves because of their efficiency (i.e., the money from the sale of their projects is enough to sustain them). According to [Abubakar \(2015\)](#) a farmer is in business only when he feeds his cow with NGN 200 per day and the cow produces milk worth NGN 400 per day. Unfortunately, native Nigerian cows cannot produce milk worth NGN 400 when fed with NGN 200. The nomadic cattle rearing system in Nigeria makes it difficult to confine them in modern cattle rearing systems. This necessitates the introduction of exotic breeds that can stay in ranches and still perform well. If this is possible, the problems of cattle rustling and clashes between cattle herdsman and farmers (which is a serious problem in Nigeria today) will be controlled to the minimum.

Importation of agricultural products that are natively produced in Nigeria has hampered agricultural growth in the country. These products are imported in bulk making them cheaper than comparable products produced in Nigeria. As a result, farmers find it very difficult to sell their products, as people prefer the cheaper foreign products. For example, tomatoes and bananas are imported from Ghana although these crops can be grown in Nigeria. Nigeria has become a dumping ground for agricultural products because of the lack of adequate government support in the form of finance, improved stock, and agricultural facilities ([Abubakar, 2015](#)). To solve this problem the importation of food items must be banned and the production and consumption of agricultural products and by-products from Nigeria should be encouraged by all means. Dangote Company is owned by a Nigerian who is the richest man in Africa. This company has just started a tomato business in Kano, which is good news for farmers as it encourages increased tomato production and provides a market for this product. Unfortunately, the establishment of this company led to a scarcity of tomatoes in 2016 (i.e., three tomatoes sold for US \$1) but nonetheless, more people are now encouraged to cultivate tomatoes because of the easy market access. Furthermore, the ease of cultivation,

and economic meltdown across the world, has led many people to turn to aquaculture to make more money. Many build concrete and plastic tanks to raise catfish as there is a high demand for the consumption and exportation of this rapidly maturing (5–6 months) fish.

Problems associated with the acquisition and distributions of fertilizers have also been identified. This problem has persisted over the years because of the selfish interest of some individuals. Fertilizer use is essential to ensure the maximum production of crops; therefore it is imperative that fertilizers be made readily available for farmers at affordable prices. Apart from the importation of fertilizers, there is also a need for the Nigerian government and private establishments to establish fertilizer companies in the country. Nigeria has all the raw materials needed to produce fertilizers; however, the only fertilizer company in Nigeria (the Federal Super Phosphate Fertilizer Company, Kaduna) was sold to another company who subsequently converted it to a warehouse ([Abubakar, 2015](#)).

There will be a drastic reduction in the importation of agricultural products if the government of Nigeria takes the bull by the horn and fixes the problems facing the agricultural sector. This will have a direct and positive impact on the economy, which is dwindling due to declining global oil prices. This change is achievable with good planning and determination to execute the plans. In summary, Nigeria must:

1. Reduce imports of agricultural produce that can be produced in Nigeria to encourage local production of these products.
2. Provide credit facilities to farmers in the form of soft loans (especially to young graduates) to help tackle unemployment, increase food production to tackle the problem of food insecurity, and boost the country's GDP.
3. Enact laws that permit land ownership by individuals. The land tenure system that allows the government to own land has hindered many private individuals who want to go into farming. This calls for a modification of this law to make land readily available for all those who want to go into crop farming or livestock production.
4. Provide good feeder roads to allow farmers to transport their produce to market with ease. Many crops spoil before they get to the city market and these items cannot be sold as a direct result of the bad roads. Providing better roads will help farmers get their goods to markets on time and reduce transportation costs.
5. Provide electricity for processing and storage of farm produce so they are available all year round. A constant electricity supply will ensure proper preservation and processing of these products.
6. Import improved breeds of crops, animals, and GMOs. Most Nigerian crop species and animal breeds have low production capabilities. Importing improved breeds to be cultivated in Nigeria or to be used to improve native breeds will ensure enhanced and improved yields.
7. Provide modern machines and infrastructure to improve the productivity of plants and animals. These should be provided to farmers or their cooperatives at subsidized prices.

8. Train farmers in modern farming practices. This is important so farmers are able to translate research results into practice in the field. Training could be organized on better management practices, new diseases, control, and modern ways of farming.
9. Improve disease prevention. This can be achieved through the provision of animal vaccines and resistant crop varieties. Good management practices to prevent disease infection could also be encouraged among farmers.

6 Awareness and Utilization of Genetically Modified Foods in Nigeria

The science of GMFs is still very new in Nigeria and most Nigerians are unaware of this technology. To date there is little or no utilization of GMFs in Nigeria. [Alarima \(2010\)](#) assessed the awareness, knowledge, and perception of GMFs in selected institutions in southwest Nigeria and found that GMF awareness varied among respondents. The internet (75.3%), newspapers (67.5%), radio (51.3%), journals (83.8%), and magazines (50.6%) were the major sources of GMF information; however, the awareness and knowledge of GMFs remained low. Furthermore, those who were knowledgeable about GMFs also supported their introduction to the Nigerian market but they were skeptical about the country's institutional preparedness for the approval and release of GM crops due to the perception of risk inherent with genetic modification. Awareness was also related to perception of the effect of genetic modification on the environment ($P = 0.21$, $P < 0.01$) and the risk of use ($P = 0.20$, $P < 0.01$). [Alarima \(2010\)](#) concluded that awareness campaigns should be performed to provide information on the benefits and risks that GMFs provide to consumers. [Fig. 8.2](#) (from the work of [Ayanwale, 2004](#)) illustrates the responses of Nigerians when questioned about the biosafety issues of GMFs. The authors found that 13% believed that they could knowingly eat GMFs whilst 20% believed that GMFs will put farmers out of business if allowed into Nigeria; 20% were willing to eat GMFs, 23% agreed that GM plants and animals were safe to eat, and 24% agreed that they were safe to grow.

[Ayanwale \(2004\)](#) presented the results of a national survey on public awareness of agricultural biotechnology from the Nigeria Agriculture and Biotechnology Project (May 2004). The study was carried out across the country using a total of 891 respondents that included students, farmers, academics, market women, politicians, businessmen, policymakers, and teachers. A pretested questionnaire was used for the exercise. The results showed that the respondents were aware of biotechnology and that the source of their awareness was stories in the media (i.e., from television and newspapers). The author also observed that the respondents did not have a clear understanding of what biotechnology was about and some were confused with the difference between biotechnology and normal breeding techniques. The results also revealed that the respondents were interested in biotechnology and they wanted it to be used to resolve the agricultural problems in Nigeria.

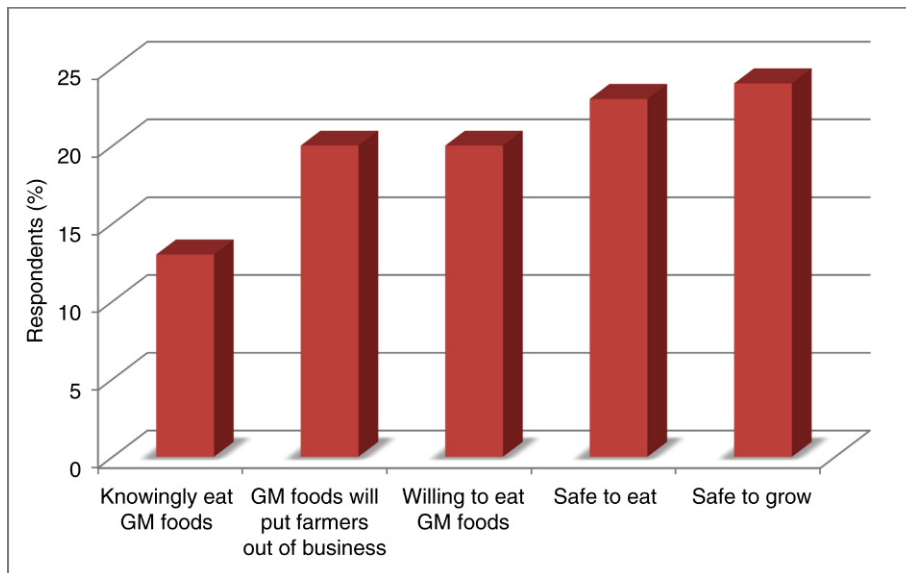


Figure 8.2: Response of Nigerians on the Biosafety Issues on GMFs. Ayanwale, A.B., 2004. *Biotechnology awareness survey in Nigeria. A draft report submitted to Nigeria Agriculture and Biotechnology Project. International Institute of Tropical Agriculture, Ibadan, Nigeria, pp. 1–19.*

Eneh et al. (2016) studied consumer food awareness in Enugu (Nigeria) and observed that GMFs are promising for food security as they are associated with higher agricultural productivity, which is especially important in the face of global warming and unstable economies and the need to improve human health and protect the environment. The applications of GMFs are increasing in developed countries and across the world. GMFs are appearing on shelves of department stores in the United States and in other industrialized countries, as well as in developing countries, such as Burkina Faso, Sudan, and Brazil. Eneh et al. (2016) aimed to estimate the level of awareness of GMFs and their sources among food consumers in Enugu, an ancient coal city of great political, commercial, and socioeconomic importance. Questionnaires were used to obtain data from randomly selected patrons of a department store. Average values were used to test the hypothesis and the results showed that 26.7% of food consumers were very much aware of GMFs, 36.7% were not very much aware, 1.6% were not sure, and 21% were not aware at all; 21.7% had heard of GMFs through newspaper articles, 13.3% through institutions, 30% from family/friends, 38.3% from the television, 33.3% from the radio, 3.33% from reports, 10% from seminars/conferences, 10% from books, 3.3% from journals/articles, and 11.3% from the internet. Increasing the publicity of GMFs was recommended to enable food consumers to make more informed choices.

Oladele and Akinsorotan (2007) performed their study in scientists between 31–40 years of age (59.40% of the total scientific population). Of these scientists, 44.44% had an MSc

degree and 89.60% had obtained understanding of GM organisms from journals. Males only accounted for 6.7% of the respondents. Most of the scientists had low awareness (52.8%) and low perception (54.5%) of the products of GM organisms. The authors also established significant relationships between the perception of GMFs and the respondent's religion, age, and source of information. However, there were no significant differences in the awareness and perception of respondents from different universities and research institutes under consideration.

In their study of the potential benefits of genetic modification, [Ademola et al. \(2004\)](#) found that 54 small-scale farmers had a very poor understanding of the genetic modification of plants and animals. The majority of respondents believed that the technology would lead to high-yielding varieties of crops and animals, shorter growing cycles, and better nutritional values; however, a few respondents expressed their concerns about the health and environmental effects of GMFs.

This research explains why it is important to identify the challenges that could affect the adoption of GM technologies. Some of the challenges to be addressed include the lack of information about GMOs due to poor extension services, the low level of education, and fears of the dangers of genetic modification. These studies emphasize that failure to proffer solutions to these challenges will hinder the implementation of GM technology in Nigeria and Ghana.

[Mathews-Njoku and Adesope \(2008\)](#) used structured questionnaires to assess the policy, awareness, and use of biotechnology products in 60 farmers. They observed that 88.3% of the respondents were aware of the existence of biotechnology products and 76.7% confirmed that they had used some biotechnology products. GM cassava was the commonest of all the biotechnology products used and this gave a mean score of 3.25. This was closely followed by GM maize and tree crops with mean scores of 2.68 and 2.67, respectively. The results also showed that GM cassava varieties were the most commonly available biotechnology products in the study area, closely followed by tree crops. The authors recommended that farmers be adequately and properly orientated about biotechnology and its related advantages and disadvantages.

In their study on GMFs for sustainable development in Africa, [Okigbo et al. \(2011\)](#) explained how the genetic modification of crossbreeding and selection, which has been practiced for decades, was meant to develop new breeds of animals and varieties of crops. However, genetic engineering can perpetuate specific traits to the next generation more rapidly and with more accuracy than traditional breeding and selection. The authors also pointed out some benefits of genetic engineering including improved fruit storage, increased food production, improved economic benefits, improved nutritional and health benefits, and improved environmental effects. Furthermore, they also explained that genetic modification allows the generation of new traits that can be introduced into animals, crops, and microorganisms.

However, they did note the concerns that the introduction of GMFs and organisms into the ecosystem could disrupt nature, which could be detrimental to the health of man and trigger the extinction of endangered plants and animal species. They recommended that public awareness campaigns be carried out on the various controversies about the genetic modification of plants and animals.

7 Some Genetically Modified Foods in Nigeria

The Federal Ministry of Environment established the National Biosafety Management Agency (NBMA) in 2015 with a mandate to ensure proper regulation of modern biotechnological activities and GM organisms so as to protect the lives of Nigerians. However, the little (or total lack of) documentation on Nigerian GMFs meant that many were unsure of what was happening. In 2016 the Minister of Environment, Amina Mohammed, emphasized that no GM organisms were officially grown in Nigeria, finally putting to rest the growing concerns of many Nigerians and leaving everyone with reasons not to panic (Ugeh, 2016).

Prior to the briefing from the Minister, a few field trials of GMFs had been approved (Table 8.1). May (2015) observed that the NBMA approved the first Nigerian trial of GM rice at the National Cereals Research Institute (NCRI) in Badeggi, Niger State. The new GM rice was reported to use nitrogen and water more efficiently than conventional rice. The agency also approved confined field trials in Umudike, Abia State for biocassava plus with provitamin A and for GM cassava that was resistant to cassava mosaic virus disease. Unfortunately the results of these trials are not available yet. Another trial regarding insect-resistant cotton is ongoing; however, insect-resistant cotton for commercial release will be subjected to further processing for the next 2 years.

Nigeria's quest for biotechnological advancement started in 2001 with the adoption of the National Biotechnology Policy and the establishment of the National Biotechnology Development Agency (May, 2015). The government of Nigeria has enjoined the people to

Table 8.1: GM crop trials in Nigeria.

	Crops	Locations	Modifications
1.	Rice	National Cereals Research Institute Badeggi (NCRI) Niger State	Efficiency in nitrogen and water use
2.	Cassava	National Root Crops Research Institute (NRCRI), Umudike, Umuahia, Abia State	Fortification with vitamin A
3.	Cassava	NRCRI, Umudike, Umuahia, Abia State	Resistant to cassava mosaic virus disease
4.	Cotton	—	Insect resistant cotton

Table 8.2: Institutes involved in biotechnology (genetic modification of foods) in Nigeria.

	Institutes	Mandates
1.	National Biotechnology Development Agency (NABDA)	Promotion, coordination, and deployment of cutting-edge biotechnology research and development, processes, and products for the socioeconomic wellbeing of the nation
2.	Federal Ministry of Science and Technology (FMST)	To facilitate the development and deployment of science and technology apparatus to enhance the pace of socioeconomic development of the country through appropriate technological inputs into productive activities
3.	National Centre for Genetic Resources and Biotechnology (NACGRAB)	To conduct research, gather data, and disseminate technological information on matters relating to genetic resources, conservation, utilization, and biotechnology applications
4.	Sheda Science and Technology Complex (SHESTCO)	To develop infrastructures and systems that will facilitate the support and enhancement of technology innovations, creation of technology-based firms, and the promotion and development of research results
5.	National Agency for Food and Drug Administration and Control (NAFDAC)	To safeguard public health by ensuring that only the right quality foods, drugs, and other regulated products are manufactured, exported, imported, advertised, sold, and used
6.	International Institute of Tropical Agriculture (IITA)	Biotechnology and genetic improvement, natural resource management, social science and agribusiness, plant production, plant health, and nutrition and food technology

cooperate with their desire to diversify the economy. [Table 8.2](#) shows some institutes involved in the production of GMFs in Nigeria.

8 Advantages of Genetically Modified Foods

Some of the advantages of GMFs according to [Panse \(2014\)](#) are as follows:

- GMFs are more productive and have larger yields than non-GM crops.
- GMFs could offer more nutrition and flavor as specific nutrients or traits could be deliberately inserted into a plant or animal (e.g., cassava fortified with vitamins).
- GMFs could be specifically designed to eliminate allergy-causing properties.
- Some GMFs have inbuilt resistance to pests, weeds, and disease, which will lead to increased productivity of the plants and animals concerned.
- Some GMFs are capable of thriving in regions with poor soil or adverse climates due to the insertion of genes that control hardiness or the ability to thrive.
- GMFs may be more environmentally friendly as they require less herbicides and pesticides, which will reduce the production and maintenance costs.
- GMFs may be more resistant to deterioration and spoilage (e.g. GM tomatoes with extended shelf lives).
- GMFs can be grown on relatively small plots of land and they provide a solution to feeding the growing global population.

9 Challenges Related to Genetically Modified Foods

Some of the challenges of GMFs according to [Panse \(2014\)](#) are as follows:

- Scientists can choose the genes that they wish to manipulate, but they can't (in most cases) choose where the gene will be inserted in the host chromosome and they have no way of controlling gene expression.
- Further research into the risks associated with GMFs should be performed. These experiments are usually difficult and expensive.
- GMFs are not labelled and therefore consumers do not know if they are buying modified foods or not.
- GMFs are seen as a risk to food diversity because only the plants or animals being propagated will be dominant.
- Herbicide- and pesticide-resistant GMFs could give rise to superweeds and superpests that would need new, stronger chemicals to destroy them.
- GM crops can cross-pollinate with nearby non-GM plants, which may create ecological problems and health issues to those who are unaware.
- GM organisms may not be the answer to world hunger and health. Instead focus should be placed on improving organic agricultural practices that are kinder to the earth and healthier for humans.

10 Controversies About Genetically Modified Foods in Nigeria

10.1 The Dangers of Genetically Modified Crops in Nigeria

[Rhodes-Vivour et al. \(2016\)](#) expressed their worry to the President of the Federal Republic of Nigeria regarding the introduction of GM crops into the food ecosystem. In March 2015 the World Health Organization classified glyphosate, the key ingredient in herbicides and pesticides, as carcinogenic. The Sri Lankan government immediately placed a ban on the use of glyphosate as they felt it was responsible for the increasing number of chronic kidney disease patients in Sri Lanka. [Rhodes-Vivour et al. \(2016\)](#) were therefore amazed that these were the exact same chemicals that the Director General of the NBMA, Mr. Rufus Ebegba, was pushing to spray on Nigerian farms. They stressed that manufacturers try to convince regulators to ignore the toxicity of their products (as was seen with bovine growth hormone in Canada and other scandals in Indonesia) in a bid to avoid environmental impact studies being conducted on cotton grown using their chemicals ([Rhodes-Vivour et al., 2016](#)).

10.2 Nigeria's Desperate Need for Export Earnings

Some groups have opined that the introduction of GMFs in Nigeria will negatively affect the economy and must be prohibited ([Rhodes-Vivour et al., 2016](#)). They emphasize that allowing the

Nigerian agriculture system to be contaminated with GMOs would leave produce (i.e., oil) largely unsold since many of the major powers that buy Nigerian products have banned GMFs. Exposing farmers and agencies to the high price of imported seeds due to the exchange rate is also likely to affect the economy and make farmers dependent on foreign companies for a primary input of production (i.e., seeds). These GM seeds have also been engineered so that they produce crops that are sterile, ensuring that farmers must buy new seeds every year, thus protecting the “Patent rights” of the company. In doing this, a primary input of agriculture that is abundant in nature becomes monopolized, commoditized, and outsourced (Rhodes-Vivour et al., 2016).

There is an increased global demand for organic foods, but whilst organic crops and animals can enter all markets, GM organisms can only go into specific markets. This must have informed the decision of President Putin who has set out his plans to make Russia the world’s largest exporter of non-GM products. Problems of contamination, crop failure, and pest and weed resistance have also been suggested to have direct effects on health and the environment. In 2011, scientists in Argentina detected long-term effects (e.g., birth defects and increased incidences of child cancer caused by glyphosate) in areas where GM crops were grown (Rhodes-Vivour et al., 2016).

10.3 Monsanto, Genetically Modified Organisms, and Nigeria’s Bleak Future

Nubari (2014) commented on Monsanto, GM organisms, and Nigeria’s bleak future using the words later. Our country Nigeria just became a member of the New Alliance for Food Security and Nutrition Initiative, an organization established by the United States and the G8. The aim of this organization is to create jobs, reduce poverty, and increase food productions. However, Nubari (2014) saw the initiative as an alliance that was meant to coerce African countries to change policies that would hinder the operation of companies, such as Monsanto in exchange for aid money. The author emphasized that by allowing GM organisms, Monsanto and other large-scale organizations in Nigeria would cause the country more loss than gain. He buttressed his point by stating that these GM companies would ask the government to change land and seed laws to favor them, which would give them control of our lands and what we choose to plant. This would force farmers to buy their seeds instead of being allowed to use the seeds indigenous to the country. This will eventually destroy our genetic resources.

Nubari (2014) questioned that if a farmer buys GM seeds from Monsanto, plants them, harvests the crop, then can’t replant part of his harvest, then is it that the crop is good enough for human consumption? The issue of dependence on these companies, which was referred to as slavery, was also raised.

10.4 Revisiting Genetically Modified Foods in Nigeria

Professor Oyewole Tomorri, President of the Nigerian Academy of Science said that genetic modification is a new aspect of science that is still being studied, and that since their debut, GM

organisms have attracted many enemies that doubt their efficacy, but this does not mean that the technology is bad or injurious to humans and the environment (Abutu, 2016). He further stated that “the challenge with GM organisms to a large extent has to do with the scientists researching on it. They have failed to communicate openly, and where there is lack of evidence based information, ignorance thrives, and that is where we have found ourselves today.”

Professor Mohammed Ishaku, lead investigator of the GM cowpea project, and one of the panelists, noted that there was nothing to fear about GMFs. “Yes, we are aware how people react to every technology that comes. Take the airplane, for instance, when it first came people were saying no, but here we are today, you can agree with me that the plane has made life a lot better and easier” (Abutu, 2016).

As a Nigerian scientist from a very poor farming background, Professor Ishaku stated that he was aware of the challenges of farmers, food challenges for rural households, and diseases that affect our crops and children. “Farming is becoming a hopeless venture for the rural populace, who invariably constitute about 70% of Nigerian farmers, because of the yearly decline in yield, which is occasioned by pests. But now we have a technology that can handle the threats posed by these pests, so why shouldn’t we go for it? I am a Nigerian and will never champion nor research on anything that will have negative impacts on the citizens” he added (Abutu, 2016).

Miriam Bassey, a lawyer and program officer with Friends of the Earth (an international nongovernmental organization campaigning for a safe environment) said that Nigeria and Nigerians should say no to GM organisms. She reasoned that the country lacked the capacity and potential to handle any risk or emergency that may arise from its deployment. She further argued that the introduction of GM crops would contaminate the natural soil and pose a risk for the entire country. She also mentioned that GM organisms introduced in India had led to the deaths of farmers after the assured bumper harvests failed to happen. However, as the debate rages as to whether Nigeria should allow GMFs, a former director general of the National Biotechnology Development Agency, Professor Bamidele Solomon, said that Nigerians were already eating GMFs. “If you eat cornflakes, because all Kellogg’s cornflakes come from *Bt* corn, then you are already eating GMFs. We all wear clothes from China, they are all made from *Bt* cotton,” he said. Solomon said rather than dissipate energy on whether we want to eat GMFs, we should channel energy into articulating a law that would guard against the misuse of the technology (Abutu, 2016).

Dr. Shehu Idris, a freelance research scientist, was in total support of Professor Solomon’s view. “There is this notorious international rating we have won for ourselves as the most travelled people in the world. Anywhere you go you will see a Nigerian and the bulk of the world’s food supply is coming from GM, which countries such as the United States have adopted. So, if you visited the United States the possibility that you have eaten GMFs is high.” He further questioned “if it is good for us to eat in the United States, why do we have

to protest against it here?” Nigeria has witnessed the influx of multinational supermarkets originating from countries where GM crops and foods are allowed. According to experts, the implication of this development is that GM products are already passing into the country through the back door (Abutu, 2016).

Okon Akparanta, a public affairs analyst, said “check out all the big cities we have and you will see the South African shops and these get their supplies from their home country. We allow them to operate here, yet we don’t want to open our doors to GM products” (Abutu, 2016).

Dr. Mark Ukwungwu, a rice researcher and head of the NCRI, said that GM technology would make farming attractive and assist the nation in overcoming the threat of food insecurity (Abutu, 2016).

10.5 Reasons Why Nigeria Must not Accept Genetically Modified Organisms

Falaju (2015) explained why Nigeria must never allow GM organisms. The author quoted Dr. Vandana Shiva, an international environmentalist and agriculturist, who warned that the introduction of GM crops to Nigeria would be another means through which the GMO originator (Monsanto) and other related western corporations could make huge profits from Nigeria and enslave farmers at the expense of their wellbeing. He explained that GM organisms are not the best science and technology for food production and the survival of Africans, and that the approach has negative impacts on the soil and human existence. According to Falaju (2015) over 300,000 farmers committed suicide in India due to the negative impact of GM organisms. He therefore used this as a basis for campaigning against Nigeria accepting GM organisms into the country. He further explained that the adoption of genetically engineered seeds and organisms, as well as the use of chemical fertilizers by African nations was suicidal because they do not deliver on their promises and only result in pain, deprivation, and death (Falaju, 2015).

Potential health concerns have been used by anti-GM advocates who emphasize that GMFs have not been adequately tested to prove their safety. The main concerns relate to negative reactions against the novel gene (Falaju, 2015). Toxic effects may also occur in people who consume crops that have been GM to be resistant to pesticides, such as *Bacillus thuringiensis* (Bt) or glyphosate.

11 Conclusions

This chapter has assessed works on the awareness and utilization of GMFs in Nigeria and other parts of the world. The importance of GMFs was explained; however, many countries (including Nigeria) have not accepted this technology because of the associated controversies.

The debate over GMFs in Nigeria is overwhelming. It currently includes intellectual property rights, the rights of farmers to plant whatever they chose, and the rights for truthful information about GMFs before the Nigerian government dives headfirst into the GMF program. Taking into account the health implications and the resultant effects of GMFs in Nigeria, proper sensitization should and must be generated by stakeholders to reach a more objective view on GMFs. Furthermore, agriculture has always been the big brother of the Nigerian economy even when oil overtook it and almost relegated it to the background. With the global downfall in the price of crude oil, there is a call to revive agriculture, as this is the only alternative source of funds for the country. However, whether to use GMFs is still controversial. The agricultural sector is the lifeline of any country that wishes to survive. Current facts about GMFs are controversial and this is enough for the Nigerian government to pause and reflect on the long-term results of GMFs. The interest of the Nigerian government in respect to the GMF policy as seen in the signing of the bill is hasty and hazy. The policy on GMFs must be generated through the choice of the people after they have been properly educated on the matter and not through a high-standing administrative directive or bill. It is important that the people are properly educated on GMF policies as the mistrust against GMFs is gathering and if care is not taken the policy will be short changed due to the inherent health implication of GMFs.

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Further Reading

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Biosensors as Advanced Device for the Transgenic Plants and Food and Detection

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1 Introduction

The development of biotechnology has improved agriculture through the introduction of genetically modified organisms (GMO), which have characteristics of agronomic interest. The International Service Acquisition of Agri-biotech Applications (ISAAA) reported that 18 million farmers cultivated GM crops on over 181.5 million ha across 28 countries in 2014. However, these GM crops are not homogenously disseminated over the world. Latin American, Asian, and African farmers cultivated 53% of the GM crops. The United States, Brazil, and Argentina are the leading producers of GM crops cultivating 73.1, 42.2, and 24.3 million ha, respectively. In Africa, South Africa, Burkina Faso, and Sudan planted 2.7, 0.5, and 0.1 million ha of GM crops, respectively. India and China recorded the highest area of GM crops in Asia with 11.6 and 3.9 million ha, respectively. In the European Union (EU), Spain, Portugal, Romania, Slovakia, and the Czech Republic planted 143,016 ha of *Bacillus thuringiensis* (*Bt*) maize; Spain was by far the largest producer, planting 92% of the total *Bt* maize hectareage (James, 2014).

Approximately 130 GMOs have been authorized for commercial use as food and/or feed. Of the total global crop area, 82% is used for soybeans, 68% for cotton, 30% for maize, and 25% for oilseed rape. These crops or by-products from these crops are also common ingredients of food and composite feeds for food-producing animals (Bawa and Anilakumar, 2013).

According to the World Health Organization “GMOs are organisms, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally” (Tagliabue, 2016). The cultivation of GMOs presents several advantages, such as enhanced yield and quality of harvests. The insertion of an exogenous DNA fragment into the

plant genome confers new crop protection traits, such as the herbicide tolerance and insect resistance.

Despite the advantages of GMO plants, their use as foods is not accepted worldwide, especially in the EU where consumers are very concerned about their safety. The main safety issues over GMOs relate to the potential gene flow to other organisms and allergenicity (Kleter and Kok, 2010). Regardless of the controversies surrounding GMOs, their cultivation is constantly increasing, either in field trials or for commercial production. The number of GM species and the countries involved, the diversity of novel genetic elements, and the global trade have also increased. All these facts mean that it is increasingly complex to detect and identify GMO-derived material (Holst-Jensen et al., 2012).

The increase of novel food production and the lack of information and public confidence in GMOs has led some governmental regulatory agencies to establish compulsory labeling requirements. Nevertheless, there is significant international heterogeneity in the labeling regulations, where countries or regions with voluntary labeling guidelines for GM food (e.g., USA, Canada, and Argentina) are opposed to those with mandatory labeling requirements (e.g., the EU, Australia, and Brazil) (Lucht, 2015).

The principal guides for the deliberate environmental release of GMOs are described in the EU directive 2001/18/EC (European Commission, 2001) and the regulations (EC) 1829/2003 and (EC) 1830/2003 and these provide the procedure for the new authorization of all food and feed derived from GMOs (European Commission, 2003a,b). Labeling is mandatory for food and feed products containing, consisting of, or produced from GMOs at levels above 0.9% (for EU-authorized GMOs). Anything below this threshold is considered to be accidental, and the foodstuff can be placed on the market without labeling. The threshold is reduced to 0.5% for nonauthorized GMOs, providing that the source has been positively preevaluated and that an adequate analytical detection technique is available on the market. For other nonauthorized GM food and feed the threshold is 0% and this may have economically important consequences since derived products are considered illegal in the EU (European Commission, 2003b).

The differences in GMO regulations between countries and the impact this has on global trade, and considering that the current methods of cultivation, storage, and transport do not permit complete segregation of authorized and nonauthorized GMOs, some coexistence must be expected. This has led to an irregular situation. As the EU does not cultivate significant quantities of cereals it is strongly dependent on imports from America; however, the EU must legally reject these imports as they contain nonauthorized GMOs (Davison, 2010).

Therefore, appropriate analytical methods are necessary to ensure that regulations are applied, consumer rights are maintained, and the presence of GMOs in crops, food, and feed is confirmed.

2 Conventional Analytical Methods for the Assessment of GMOs

Monitoring GMOs has numerous purposes. Qualitative analysis can be used to detect GMOs or to discriminate between authorized and nonauthorized GMOs, whereas quantitative analysis can be used to verify the labeling compliance in processed food and feedstuffs (Holst-Jensen, 2009).

Both qualitative and quantitative GMO assessments have been based on the detection of two different biomolecules: protein and DNA (Fig. 9.1). Protein-based methods detect the presence of novel proteins encoded by the DNA insertion and DNA-based methods detect the presence of a DNA sequence introduced into the plant genome (Manzanares-Palenzuela et al., 2015).

2.1 Protein-Based Methods for the Detection of GMOs

Protein-based detection methods are mainly immunological assays, which rely on the use of specific monoclonal or polyclonal antibodies for immunochemical detection (Michellini et al., 2008). Both monoclonal (highly specific) and polyclonal (highly sensitive) antibodies can be used depending on the amount needed, the desirable specificity, the time frame, and the

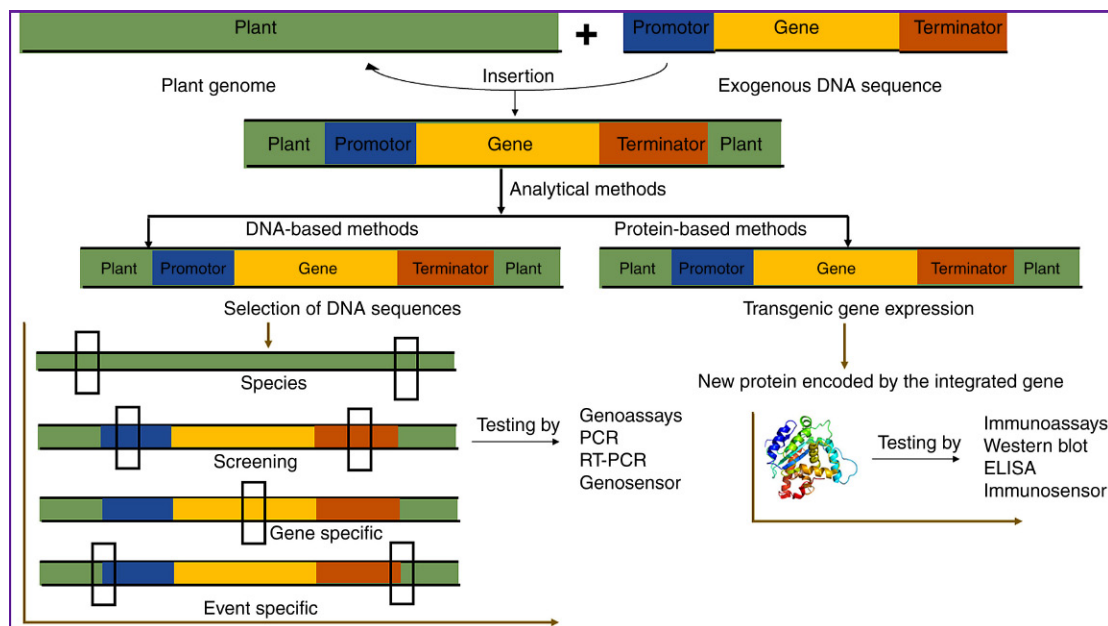


Figure 9.1: Analytical and Biological Perspectives of Protein and DNA Detection.

Adapted from Holst-Jensen, A., Bertheau, Y., de Loose, M., Grohmann, L., Hamels, S., Hougs, L., Morisset, D., Pecoraro, S., Pla, M., Van den Bulcke, M., Wulff, D., 2012. Detecting unauthorized genetically modified organisms (GMOs) and derived materials. *Biotechnol. Adv.* 30, 1318–1335.

cost (Ahmed, 2002). Immunological assays are available for the detection and quantification of proteins expressed by most biotechnology-derived crops in commercial production.

The most prominent examples of these assays include lateral flow devices, enzyme-linked immunosorbent assays (ELISAs), one-dimensional SDS gel electrophoresis, and western blotting (Ahmed, 2002; Michelini et al., 2008). Immunoassays are normally performed in one of two formats: competitive or noncompetitive assays. Competitive immunoassay use a label-free analyte (e.g., antigen) extracted from a sample to compete with a labeled analyte for specific antibody binding. Noncompetitive assays use an unlabelled analyte to bind to a labeled antibody. These types of assays are usually performed in sandwich format, with the analyte being sandwiched between a capture antibody and a labeled antibody (Ahmed, 2002).

The ELISA methodology presents significant advantages over the other immunoassays because it is quantitative, highly sensitive, economical, high throughput, and is suitable for quantitative high-volume laboratory analysis (Ahmed, 2002).

Kamle and collaborators developed a simple, specific, and sensitive ELISA for the quantitative assessment of Cry2Ab in GM crops. Cry2Ab is one of the two proteins (the other is the Cry1Ac protein) expressed by cotton event-specific MON15985 (Bollgard II cotton). This sandwich ELISA was developed in a 96-well microtiter plate using a polyclonal rabbit IgG as a capture antibody and a monoclonal mouse anti-Cry2Ab-IgG as the labeled antibody. The horseradish peroxidase (HRP) enzyme was used as a signal for the monoclonal antibody, which produced a fluorescent signal (brilliant blue color; maximum emission at 450 nm) when in contact with the substrate 3,3',5,5'-tetramethyl benzedine (TMB) and hydrogen peroxide (H₂O₂). Polyvinylpyrrolidone (2%) and dithiothritol (1%) were used to extract the transgenic protein (analyte) from the GM cottonseeds to ensure minimal interference with the ELISA assay. This methodology had a limit of detection (LOD) of 1 pg/g of cottonseeds and importantly it did not show any cross-reactivity with other GM proteins (Cr1Ab, Cry1Ac, EPSPS, or Vip) (Kamle et al., 2011).

A similar ELISA technique has also been developed for the quantification of Cry1Ac in *Bt* transgenic crops. In this protein-based assay a LOD of 100 ng/g of transgenic leaf samples was obtained (Kamle et al., 2013).

Hybridoma technology is used to produce large amounts of monoclonal antibodies for use in ELISAs. This process begins by injecting an antigen into a mouse, which induces an immune response and results in the production of antibodies in this animal; however, obtaining monoclonal antibodies using this technique is difficult and tedious. An alternative to hybridoma is antibody-engineering technology (i.e., phage antibody display technology) (Pande et al., 2010). Using phage display technology it is possible to generate monoclonal single-chain variable fragments (scFvs) with the desired binding affinities and specificities (Garet et al., 2010).

This method was used to construct a naïve mouse phage display library to generate scFvs against the transgenic Cry1Ab toxin. Positive phage scFvs were isolated, sequenced, and characterized using ELISA. The new methodology presented a LOD of 0.008 mg/mL and linear range between 0.018 and 6.23 mg/mL. This methodology also showed promise in detecting Cry1Ab toxin in agricultural and environmental samples (Zhang et al., 2014).

Enzymes are usually used to signal proteins in ELISA assays; however, the developments in nanotechnology have meant that new nanomaterials with fluorescent activity are being synthesized constantly. Quantum dots (QDs) are nanocrystals (luminescent inorganic fluorophores) that have a narrow emission spectra, a broad excitation spectra, and long-term photostability. Therefore, QDs could be used for a variety of analytical purposes, such as cellular labelling or immunoassays. Based on this idea, Zhu et al. (2011) developed a highly sensitive QD-based fluorescence-linked immunosorbent assay (QD-FLISA) to detect Cry1Ab in MON810 maize. Red QDs (emission at 655 nm) were used as the fluorescent marker, and were conjugated with a goat antirabbit secondary antibody. This QD-FLISA showed a LOD of 2.956 pg/mL, which was more sensitive than the conventional sandwich ELISA.

These protein-based methods are rapid and easy to use, but they do not allow the unequivocal identification of a GMO. They can only identify the presence of a transgenic protein and do not allow the user to differentiate between events that contain the same transgenic construction. For this reason these methods can be used as a convenient and cost-effective screening tool, especially for lowly expressed or unprocessed materials (Arugula et al., 2014).

2.2 DNA-Based Methods for the Detection and Quantification of GMOs

DNA-based assays are typically applied by global traders, the food processing industry, and law enforcement authorities (Holst-Jensen, 2009). It is important to note that GM crops contain an additional trait encoded by the introduced gene, and therefore transgenic food and feed (raw or processed) can be identified by testing for the presence of this gene. A typical transgene is composed of three elements (Fig. 9.1): the promoter element (usually P35S), which drives the expression of the inserted gene; the gene that of interest, which encodes a specific protein; and the terminator element (usually TNOS), which functions as a stop signal when the inserted gene is being read (Holst-Jensen, 2009; Michelini et al., 2008).

DNA-based GMO analyses can be categorized based on their level of specificity (Fig. 9.1). The least specific method targets the promotor and terminator DNA elements, which are usually present in all GMOs; the gene-specific method detects part of the inserted trait gene; and the highest specificity is achieved when the target is the unique point at which the inserted DNA and the recipient DNA cross (Arulandhu et al., 2016; Gašparič et al., 2010; Holst-Jensen, 2009; Holst-Jensen et al., 2012; Manzanares-Palenzuela et al., 2015).

DNA-based methods can be performed using techniques, such as polymerase chain reaction (PCR), real-time PCR (RT-PCR), ligase chain reaction, nucleic acid sequence-based amplification, fingerprinting techniques (e.g., restriction fragment length polymorphism), random amplified polymorphism DNA, and probe hybridization (Arugula et al., 2014). PCR and RT-PCR are the most widely used techniques for raw materials and processed foods due to their high specificity and stability in qualitative and quantitative measurements (Arugula et al., 2014; Kamle and Ali, 2013). Several steps should be considered in a DNA-based assay; DNA must be extracted from the sample (sampling should have been carried out in a manner that ensures a statistically representative sample), purified, and amplified using PCR or RT-PCR (Ahmed, 2002; Kamle and Ali, 2013; Michelini et al., 2008). State of the art PCR-based methods allow the detection and quantification of several DNA transgenic elements, such as the cauliflower mosaic virus (CAMV) 35S promotor, *NOS* terminator, *Cry* gene, and several event-specific genes (Holst-Jensen et al., 2012; Michelini et al., 2008).

The simultaneous detection and quantification of transgenic elements is of high interest for routine analysis (Michelini et al., 2008). In this sense, multiplex PCR assays have been developed for the simultaneous amplification of several genes in a single PCR reaction (Kamle and Ali, 2013). Kamle and collaborators developed a qualitative multiplex (quadruplex) PCR assay, which had the ability to simultaneously target the *Cry2Ab* gene (326 bp), the marker gene *NptII* (215 bp), the promotor *P35S* (195 bp), and the terminator *TNOS* (180 bp). This assay showed a detection sensitivity of 0.1% (below the EU limit of 0.9%) and had several advantages over the standalone common PCR assay for breeding, labelling, and establishing quality assurance protocols (Kamle et al., 2011).

Randhawa et al. (2010) also developed a qualitative and quantitative multiplex PCR technique for the detection of *CryIAc*, *CryIAb*, *Cry2Ab*, *P35S*, and *NptII*. The sensitivity of their assay was found to be 0.01%, which presented a good strategy for the assessment of GMOs in *Bt* brinjal, rice, cauliflower, potato, and okra.

A quantitative multiplex assay has been developed for the assessment of 12 EU-authorized GM maize varieties (DAS1507, DAS59122, GA21, MIR162, MIR604, MON810, MON863, MON89034, NK603, T25, Bt11, and MON88017) using droplet digital PCR (ddPCR). This method achieved a wide dynamic range, a lower limit of quantification (below 50 target copies per reaction), and an upper limit of 100,000 target copies (Dobnik et al., 2015).

Although the protein- and DNA-based assays of ELISA or PCR are conventional and typically used as reference methodologies, they are not without their own drawbacks (i.e., laborious, expensive, and time consuming) (Kamle and Ali, 2013). Therefore, efforts have focused on developing low-cost methods that generate simple and fast measurements and easy to interpret data. Bioanalytical methods that have the potential for automation and microfabrication and are based on simple and portable detection systems (i.e., biosensors) represent a valuable tool for the detection and quantification of GMOs (Arugula et al., 2014).

3 Biosensor Development for the Assessment of GMOs

The International Union of Pure and Applied Chemistry defined a biosensor as an integrated receptor–transducer device, which is capable of providing selective quantitative or semiquantitative analytical information using a biological recognition element (Thevenot et al., 1999). This dual configuration permits the quantitative assessment of the interaction between the analyte and the immobilized biocomponent. Efficient immobilization of the biomolecule on a physical transducer can be achieved using adsorption, entrapment, encapsulation, covalent binding, cross-linking, or affinity binding (Sassolas et al., 2012). Several types of surface platforms can be used in this process, namely, carbon and gold materials, which are normally modified by nanomaterials [carbon nanotubes (CNTs) or gold nanoparticles (NPs)] to increase the selectivity and sensitivity of the methodology (Barroso et al., 2011).

Biosensors can be classified by the nature of the receptor (recognition element) or the transduction process (analytical signal generation). Typical receptors include enzymes (enzymatic biosensors), microorganisms (microbial biosensors), antibodies (immunosensors), nucleic acids (DNA- or RNA-based biosensors), and detectors of the DNA hybridization reaction (genosensors). The bioanalytical signal generated by the biological reactions can be electrochemical, piezoelectrical, optical, or calorimetric (Fig. 9.2) (Ricci et al., 2007). Using biosensors with electrochemical transduction to may be a useful alternative technique for determining GMOs. Of all the biosensors the electrochemical one is the most common as it has high sensitivity, specificity, and fast analysis. Electrochemical biosensors are used

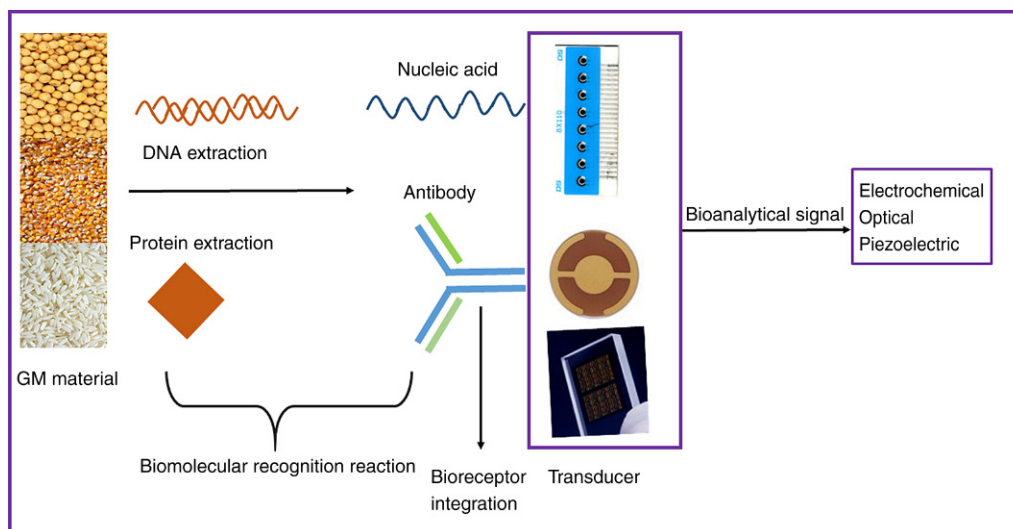


Figure 9.2: Generic Scheme of GMO Detection Using Genosensors and Immunosensors.

in point-of-care devices because they are portable, simple, easy to use, cost-effective, and (usually) disposable (Manzanares-Palenzuela et al., 2015).

The construction of biosensors for the determination of GMOs is based on detecting the inserted DNA or detecting the new encoded protein (Arugula et al., 2014). In this sense the most useful and practical biosensors for GMO analysis are the immuno- and genosensors.

3.1 Development of Immunosensors for the Detection of GMOs

The principles of immunosensors are essentially the same as for the protein-based assays, and therefore this technique is based on the specific immunochemical recognition between an antibody (or antigen) immobilized on a transducer surface and an antigen (or antibody) present in the sample (Fig. 9.3). This immunochemical reaction produces an analytical signal, which varies with the analyte concentration (Ricci et al., 2007). Depending on the transducer process, proteins (antibody or antigen) can be labeled with enzymes (e.g., HRP in electrochemical sensors), fluorophores (e.g., QDs in fluorescent sensors), or are left label free for impedimetric, optical (surface plasma resonance: SPR), and piezoelectric detection. The most developed immunosensors for the detection or quantification of GMOs are based on competitive or sandwich assays, and some examples are provided in Table 9.1.

Volpe et al. (2006) developed an immunomagnetic electrochemical sensor to detect the transgenic protein Cry1Ab and Cry1Ac in GM *Bt* maize samples. The immunoassay design

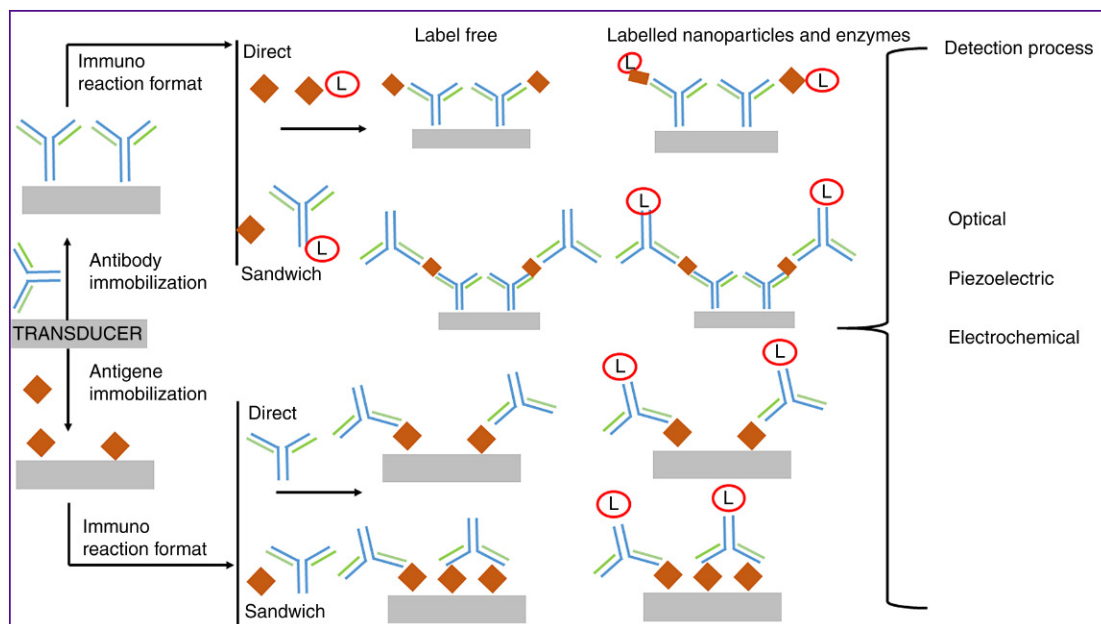


Figure 9.3: Generic Scheme of an Immunosensor Construction.

Table 9.1: Application of immunosensors for transgenic protein detection.

Transgenic Protein	Samples	Transducer	Label	Detection	Dynamic Range	References
Cry1Ab and Cr1Ac	<i>Bt</i> maize	Magnetic bead as a platform for SPCE	Alkaline phosphatase Substrate: 1-naphthyl phosphate	Electrochemical: DPV	LR: 0.25–4 ng/mL LOD: 0.1 ng/mL	Volpe et al. (2006)
EPSPS, NPII, and PAT	Soybean	Glass slide	HRP	Electrochemiluminescence	1% of GMO	Jang et al. (2011)
Cry1Ac	Soybean	Fe ₃ O ₄ @Au as a platform Glassy carbon electrode	Glucose oxidase Substrate: glucose, luminol, and H ₂ O ₂	Electrochemiluminescence	LR: 0–6 ng/mL LOD: 0.25 pg/mL	Li et al. (2013)
Cry1Ab	Maize	Glassy carbon electrode	HRP Substrate: PANI	Electrochemical: DPV	LR: 0.1–1000 ng/mL LOD: 0.07 ng/mL	Zhu et al. (2015)
Cry1Ab	<i>Bt</i> maize	Gold slide	Label free	Optical: SPR	LR: 10–500 ng/mL LOD: 5.0 ng/mL	Ming et al. (2015)
Cry1Ac	Cotton	CM5 sensor chip	Label free	Optical: SPR	LOD: 0.1 nmol/L	Zhao et al. (2013)

DPV, Differential pulse voltammetry; HRP, horseradish peroxidase; LOD, limit of detection; LR, linearity range; PANI, polyaniline; SPCE, screen printed carbon electrode; SPR, surface plasma resonance.

used a antibody sandwich, where the protein (analyte) was sandwiched between a monoclonal antibody and a polyclonal antibody. The latter was labelled with an alkaline phosphatase, which generated an electrochemical signal by differential pulse voltammetry (DPV) in the presence of 1-naphthyl phosphate (substrate). The process was scanned in the potential range of 0–600 mV.

The voltammetric signal was directly proportional to the concentrations of Cry1Ab and Cry1Ac proteins. This immunoassay construction was assembled onto magnetic beads and placed (with the help of an external magnet) onto the surface of a screen-printed carbon electrode (SPCE). This methodology was used to detect Cry1Ac protein in maize and certified reference materials. This complex procedure showed a LOD of 0.1 ng/mL, a working range of 0.25–4 ng/mL, and a total analysis time of 3 h (Volpe et al., 2006). The performance of this methodology (assessed by the LOD, linear range, and analysis time) was comparable with commercial spectrophotometric kits (Ricci et al., 2007).

Electrochemiluminescence immunosensors were also developed for the detection of recombinant marker proteins (e.g., EPSPS, NptII, and PAT) in real samples (Jang et al., 2011; Li et al., 2013).

Jang et al. (2011) used a glass slide as a solid surface matrix for the immobilization of biotinylated monoclonal capture antibodies. A sandwich assay was performed with the target proteins being sandwiched between the immobilized antibodies and HRP-labelled polyclonal antibodies. A miniature sensor (photodiode) was attached by physical contact at the top of this immunosensor assay to detect the light signal generated during the immunological reaction. This type of sensor can detect at the upper range of the GMO threshold (1%–5%). Moreover, this novel sensing device permits the simultaneous screening of multiple analytes (EPSPS, NptII, and PAT) in a single sample. It is also portable, which is important for in situ analysis.

Li et al. (2013) used core-shell $\text{Fe}_3\text{O}_4@\text{Au}$ magnetic nanoparticles (MNPs) as a platform in an electrochemiluminescence immunosensor to detect *Bt* Cry1Ac protein in soybean. The construction of the immunosensor consisted of immobilizing a primary antibody (anti-Cry1Ac) onto $\text{Fe}_3\text{O}_4@\text{Au}$ MNPs. The antigen (analyte) and glucose-oxidase labelled secondary antibody were subsequently assembled in a sandwich-type interaction. The magnetic immunoassay was attached to a glassy carbon electrode (GCE) using a magnet. Electrochemiluminescence was generated and detected by the reaction of luminol and H_2O_2 (derived from the enzymatic reaction) in the presence of glucose (a substrate of glucose oxidase). The magnetic assay showed a good sensitivity (LOD of 0.25 pg/mL) and a wide linear range (0–6 ng/mL).

Traditional monoclonal antibodies used in detection systems are very expensive and difficult to mass-produce for commercial purposes. An alternative approach could be to

use single-domain or variable-domain heavy-chain only antibodies (VHH) or nanobodies derived from the heavy chain antibody in camels. Compared with the conventional antibodies, nanobodies present excellent stability and recognize more inaccessible and cryptic sites (Zhu et al., 2015).

Zhu et al. (2015) described an electrochemical immunoassay for the detection of Cry1Ab, in which HRP-labelled nanobody was enzymatically electrocatalyzed by the deposition of polyaniline (PANI). The authors used an immune phage display library to select two nanobodies. A biotinylated primary nanobody and a HRP-labelled secondary nanobody were assembled in a sandwich assay using HRP as the signal and aniline as a substrate (which is converted to PANI). The product of the immunoreaction (PANI) was detected by DPV at 40 mV and this immunoassay was found to have an analytical range of 0.1–1000 ng/mL and a LOD of 0.07 ng/mL. Although this sensor is promising for practical applications because of its sensitivity, specificity, and robustness, it remains time consuming due to its preparation and construction.

Label-free optical biosensing techniques have become attractive for measuring antigen–antibody interactions as they permit label-free and time-resolved detection. SPR technology is a well-established, label-free technique, which has demonstrated enormous potential for the sensitive and real-time monitoring of interactions between biomolecules (Arugula et al., 2014; Ricci et al., 2007). Two novel optical immunosensors were developed to assess *Bt* Cry1Ab protein using SPR as the detection system and gold sensor chips as the transducer. The design format consisted of modifying the sensor surface with Au–Ag alloy NPs, and using thiols (1,6-hexanedithiol and 3-mercaptopropionic acid), protein A (only in sensor 2), and Cry1Ab monoclonal antibodies. The Au–Ag alloy NPs were applied to block the sensor surface and increase the sensitivity while protein A was immobilized to control the orientation of the antibodies. Both immunosensors showed a wide linear range; however, sensor 2 showed a larger detection range (linear range of 8–1000 ng/mL and a LOD of 4.8 ng/mL) due to the immobilization of protein A on the sensor surface (Ming et al., 2015).

Zhao et al. (2013) also used SPR technology to develop a label-free immunosensor for the detection of transgenic Cry1Ac in cotton. In this protein-based optical detection system a CM5 sensor chip was used as the transducer surface. Monoclonal anti-Cry1Ac antibodies were immobilized on this chip in order to construct an immunosensor. Transgenic proteins in cotton samples were detected at sensitivities lower than 0.1 nM.

Immunosensor devices are sensitive, specific, and fast, and they allow researchers to trace the presence of a transgenic protein; however, they cannot discriminate between GM ingredients that express the same or similar proteins (Manzanares-Palenzuela et al., 2015). Therefore, genosensors appear to be the method of choice for unequivocal GMO identification.

3.2 Development of Genosensors for the Determination of GMOs

Nucleic acid biosensors (genosensors) are based on the natural and spontaneous affinity of single stranded DNA (ssDNA) for its complementary strand. Procedures based on the DNA complementary hybridization reaction have been used to detect and quantify DNA in food, environmental, and clinical samples (Paniel et al., 2013).

The construction of a genosensor involves several steps (Fig. 9.4) including transducer selection, ssDNA immobilization on the transducer surface, hybridization, labelling, and analytical signal measurement (Manzanares-Palenzuela et al., 2015).

The analytical transducer process (electrochemical, optical, or piezoelectric) will dictate the type of physical surface that can be used (carbon, glassy carbon, or carbon paste). Carbon-based surfaces are mostly used in electrochemical processes whereas gold surfaces (gold electrodes, gold thin-film chips, and gold quartz crystal microbalance (QCM) chips) have been successfully used in electrochemical, optical, and piezoelectric techniques (Arugula et al., 2014; Chen et al., 2012). Gold surfaces provide advantages in term of their ability to immobilize biomolecules and form monolayers and the highly ordered nature of their monolayer (Conde et al., 2014; Paniel et al., 2013).

ssDNA immobilization is crucial as it determines the overall performance of the genosensor. Many strategies can be used to perform efficient DNA immobilization including physical adsorption, covalent binding, noncovalent affinity interaction, self-assembled monolayers (SAMs), and electropolymerization (Civit et al., 2012).

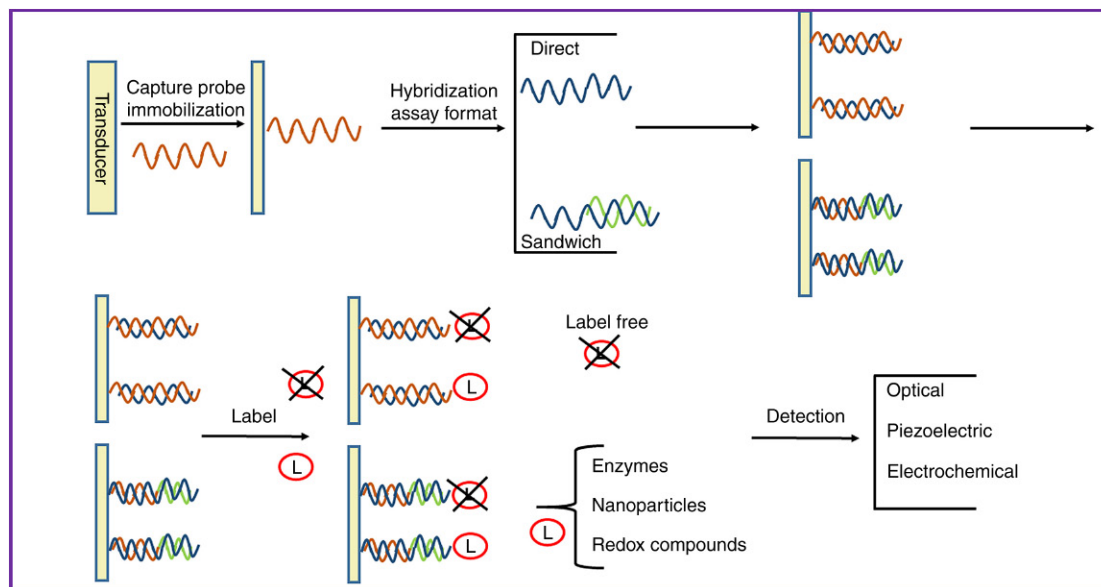


Figure 9.4: Generic Scheme of a Genosensor Construction.

The physical adsorption of DNA onto the surface of a transducer is a simple method performed using electrostatic forces; however, one drawback of this method is the low stability and desorption from the sensor surface (Paniel et al., 2013).

The high affinity between biotin and avidin can be exploited for ssDNA immobilization. Avidin (or streptavidin) is usually immobilized onto the sensor surface (through physical adsorption or chemical bonding) and a biotinylated ssDNA can be attached to the avidin binding sites. This technique provides a well-oriented ssDNA and increases the amount of DNA on the sensor surface (Manzanares-Palenzuela et al., 2015; Miranda-Castro et al., 2009).

Another strategy for ssDNA immobilization involves the chemisorption of thiolated compounds or thiolated ssDNA onto the surface of gold sensors through the formation of SAMs. The formation of a gold–sulfur layer produces a well-defined and organized surface. Covalent attachment of ssDNA to the transducer also provides a structural flexibility and increases the hybridization efficiency. In this instance, ssDNA with an amine or carboxyl terminal functional group is chemically linked (usually using carbodiimide as a mediator) to a COOH or NH₂ group on a previously carboxylated or aminated surface (Ariga et al., 2012; Bang et al., 2016; Paniel et al., 2013; Radi, 2011).

The high electrical conductivity, useful chemical and physical proprieties, and long-term stability of nanomaterials (e.g., gold NPs and CNTs) have seen them be used in the production of enhanced sensor surfaces, which probably increases the analytical detection signal (Martínez-Paredes et al., 2010).

The next step in the construction of a genosensor involves selection of the hybridization reaction format. Direct hybridization assays and sandwich assays are the most common forms of hybridization. Direct hybridization involves the direct binding (single site binding) of two complementary DNA strands while sandwich hybridization involves double-site binding. Targets are initially recognized by a signaling ssDNA (performing a first hybridization reaction) then a second complex binding takes place when the mix comes into contact with a capture probe (usually immobilized in a physical substrate). Quantitative and qualitative detection of the hybridization reaction can be performed using several labels (usually linked to a signaling ssDNA), such as enzymes (e.g., HRP and POD) or metals (e.g., Au and Ag).

The most reported genosensors for the detection of transgenes are screening methods (the least specific) that target P35S and TNOS. Table 9.2 provides examples of genosensors that have been used to detect and/or quantify GMOs.

Carpini et al. developed an electrochemical genosensor to detect sequences of the transgenic *P35S*. The genosensor was integrated in screen-printed gold electrodes by immobilizing a mixed monolayer consisting of 25-mer thiol-tethered DNA and a spacer thiol (6-mercapto-1-hexanol). The hybridization reaction was electrochemically detected using an enzyme-amplified scheme based on linking of a streptavidin-alkaline phosphatase conjugate. The

Table 9.2: Genosensors developed for the detection of GMOs.

DNA Target	Samples	Transducer	Label	Detection	Dynamic Range	References
P35S	Synthetic DNA and PCR-amplified material	SPCE	Alkaline phosphatase Substrate: α -naphthyl phosphate	Electrochemical: DPV	LR: 0–25 nmol/L LOD: 0.25 nmol/L	Carpini et al. (2004)
P35S	Soybean	GCE and PtNPs	[Co(phen) $_3$] $^{3+}$	Electrochemical: DPV	LR: 2.14–214 nmol/L LOD: 1.0 mol/L	Mao-Qing et al. (2008)
P35S	Synthetic DNA	GCE	PbSNP	Electrochemical: DPASV	LR: 0.012–48 nmol/L LOD: 4.38×10^{-3} nmol/L	Sun et al. (2008)
P35S	Synthetic DNA	Au interdigital	Label free	QCM and EIS	LR: 0.025–0.08 nmol/L	Lien et al. (2010)
P35S	Certified material	SPE modified microspheres and AuNP	Anthraquinone	Electrochemical: DPV	LR: 2×10^{-6} –2 nmol/L LOD: 7.79×10^{-7} nmol/L	Ulianas et al. (2014)
P35S and TNOS	Synthetic DNA	AuE	CdSNPs	Electrochemical: DPASV	LR: 0.008–4 nmol/L LOD: 0.00275 nmol/L	Sun et al. (2007)
<i>Bt</i>	Synthetic DNA	AuE	AgNP	Electrochemical: solid state voltammetry	LOD: 1×10^{-5} nmol/L	Jiang et al. (2011)
<i>Bt</i>	Synthetic DNA	SPCE	Label free	EIS	LR: 0.001–1000 nmol/L LOD: 1×10^{-5} nmol/L	Bonanni et al. (2009)
PEP	Synthetic DNA	GCE modified with Au–PtNPs and Pty	Label free	EIS	LR: 0.001–100 nmol/L LOD: 3.6×10^{-4} nmol/L	Yang et al. (2012)
<i>SSIIb</i> , <i>ivrp</i> , <i>Cry1Ab</i> , and MON810	Synthetic DNA	AuE	Osmium tetroxide bipyridine	Electrochemical: SWV	LR: 25–200 nmol/L	Duwensee et al. (2009)
<i>IVRP</i> , <i>SSIIb</i> , and MON810 <i>cry1a/b</i>	Transgenic maize	AuE	Osmium tetroxide bipyridine	Electrochemical: SWV	—	Mix et al. (2012)
MON810 event	Transgenic maize	ERG–CILE	Methylene blue	Electrochemical: DPV	LR: 0.01–1000 nmol/L LOD: 4.52×10^{-3} nmol/L	Sun et al. (2014)
P35S, <i>G1B1</i> , <i>CP4-EPSPS</i> , <i>PAT</i> , <i>cordapA</i> , <i>lectin</i> , and <i>SSIIb</i>	Transgenic soybean	AuE array chip	GOx and HRP Substrate: TMB and glucose	Chronoamperometry	LR: 0–20 μ mol/L LOD: 225 pmol/L	Liao et al. (2013)

A2704-12	Transgenic soybean	ERG-CILE	Methylene blue	Electrochemical: DPV	LR: 1×10^{-3} – 2×10^3 nmol/L LOD: 2.9×10^{-4} nmol/L	Sun et al. (2013)
P35S	CRM soy	Sensor chip	Label free	Optical: SPR	LR: 1–100 nmol/L LOD: 1 nmol/L	Mariotti et al. (2002)
<i>Bt11</i> , <i>Bt176</i> , <i>GA21</i> , <i>MON810</i> , <i>NK603</i> , <i>T25</i>	CRM	Optical silicon thin-film chips	HRP Substrate: TMB	Optical	—	Bai et al. (2010)
Lectin and RR	RR soybean	SA sensor chips	Label free	Optical: SPR	—	Feriotto et al. (2002)
P35S	Tobacco plants	Gold quartz crystal	Label free	Piezoelectric: QCM	LR: 0.25–1.0 ng/μL	Karamollaoglu et al. (2009)
<i>Cry1ab</i> gene	Food samples and CRM	Gold quartz crystal	Label free	Piezoelectric: QCM	LR: 0%–5% GM content	Passamano and Pighini (2006)
<i>EPSPS</i> gene	RR soybean	Gold quartz crystal	Label free	Piezoelectric: QCM	—	Stobiecka et al. (2007)
P35S and TNOS	CRM soybean	Gold quartz crystal	Label free	Piezoelectric: QCM	LR: 0–0.5 μmol/L	Minunni et al. (2001)
P35S and TNOS	GM tobacco	Magnetic beads as a platform for the PtE	Ruthenium(II) tris-bipyridine and tripropylamine	Electrochemiluminescence	LR: 5–5 μmol/L	Zhu et al. (2010)

AgNP, Silver nanoparticles; AuE, gold electrode; CdSNP, cadmium sulfide nanoparticles; CRM, certified reference material; DPASV, differential pulse anodic stripping voltammetry; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; ERG-CILE, electrochemically reduced graphene-carbon ionic liquid electrode; GCE, glassy carbon electrode; GOx, glucose oxidase; HRP, horseradish peroxidase; LOD, limit of detection; LR, linear range; PbSNP, lead sulfide nanoparticles; PtE, platinum electrode; PtNPs, platinum nanoparticles; QCM, quartz crystal microbalance; SPCE, screen printed carbon electrode; SPR, surface plasma resonance; SWV, squarewave voltammetry; TMB, 3,3',5,5'-tetramethyl benzedine.

alkaline phosphatase enzyme catalyzed the hydrolysis of the electroinactive naphthyl phosphate to naphthol, which is electroactive and detected by DPV. The genosensor response was found to correlate with the DNA target concentration at 0–25 nM with a LOD of 0.25 nM. The genosensor was used to detect *P35S* in amplified PCR samples (Carpini et al., 2004).

Mao-Qing et al. developed a genosensor by depositing platinum NPs (PtNP) onto the surface of a GCE. Compared with a standard GCE, the PtNP–GCE exhibited a higher mass-specific surface area and a higher binding activity for ssDNA. This chemically-modified electrode was then used as a platform to develop a DNA-based sensor for the detection of *P35S*. The hybridization reaction was electrochemically detected using $[\text{Co}(\text{phen})_3]^{3+}$ ion (phen is 1,10-phenanthroline) as an electrochemical indicator. Squarewave voltammetry (SWV) was used to identify a relationship between the DNA concentration and the peak intensity. The electrochemical responses ranged between 2.14×10^{-9} and 2.14×10^{-7} mol/L of target DNA and the LOD was 1.0×10^{-9} mol/L. This genosensor has been successfully used to detect *P35S* in GM soybean (Mao-Qing et al., 2008).

Lead sulfide (PbS) NPs were used by Sun et al. as ssDNA labels for the electrochemical detection of *P35S* from CaMV. The PbSNPs were synthesized in aqueous media and modified with mercaptoacetic acid (MAA) to facilitate linkage with the *P35S* DNA probe. The DNA targets were then covalently linked to a monolayer composed of a self-assembled MAA on a gold electrode (AuE) so that DNA hybridization of the target and probe occurred on the electrode surface. The volume of PbSNPs was proportional to the yield of the hybridization reaction and its assessment was carried out by dissolving the NPs with HNO_3 and measuring using a sensitive voltammetric technique (i.e., differential pulse anodic stripping voltammetry). The electrical results had a linear relationship with the concentration of target ssDNA in the from 1.2×10^{-11} to 4.8×10^{-8} mol/L and a LOD of 4.32×10^{-12} mol/L. The genosensor was able to distinguish between complementary, noncomplementary, and three-base mismatched ssDNA sequences (Sun et al., 2008).

Ulianas et al. (2014) developed a novel electrochemical genosensor to detect the *P35S* promotor from CaMV in soybean using acrylic microspheres and AuNPs coated onto SPCEs. Covalent bonding was used to immobilize the *P35S* ssDNA probe onto the modified SPCE. Hybridization between two complementary *P35S* ssDNA was measured using voltammetric techniques (namely, DPV) using anthraquinone-2-sulfonic acid as the redox indicator. A relationship between the electrochemical peak intensity and DNA concentration was found from 2×10^{-15} to 2×10^{-9} mol/L and a LOD of 7.79×10^{-16} mol/L was observed. Therefore, this biosensor is useful for the assessment of DNA from genetically modified soybean samples. The method was also reproducible and showed electrochemical stability for up to 45 days when stored at 4°C. Another advantage of this biosensor was the ability to reuse the genosensor for several times following regeneration with mild sodium hydroxide.

Sun et al. (2007) used MAA-modified cadmium sulfide (CdS) NPs (synthesized in aqueous solution) as an oligonucleotide label for the voltammetric detection of the *TNOS* gene sequence. The carboxyl functional groups present in the modified CdSNP can be covalently linked to an aminated (NH_2) ssDNA probe via N' -ethylcarbodiimide and N -hydroxysuccinimide chemical reactions. The target ssDNA was immobilized onto AuEs via covalent linking to a SAM and the hybridization reaction was accomplished on the AuE. The CdSNPs were dissolved in acid media (HNO_3) and electrochemically detected by DPV. A higher sensitivity and lower LOD (when compared with the PbSNP) were found when using CdS as an amplifying signal. *TNOS* DNA was detected at 8.0×10^{-12} to 4.0×10^{-9} mol/L with a LOD of 2.75×10^{-12} mol/L (Sun et al., 2007).

Jiang et al. (2011) reported a novel ultrasensitive electrochemical genosensor for the detection of transgenic *Bt* DNA using silver (Ag) NPs as a label. The construction of this genosensor consisted of anchoring thiolated ssDNA (SH-DNA) capture on a AuE surface. Target ssDNA was then hybridized (in a first hybridization reaction) with the SH-DNA capture before a second complex hybridization was performed with a DNA probe functionalized with a biotinylated AgNP and the target DNA. AgNP exhibited excellent electroactive properties in the solid-state Ag/AgCl process and was successfully applied to transgenic *Bt* DNA showing a LOD of 10 fmol/L (Jiang et al., 2011).

Multiwalled carbon nanotubes (MWCNTs) have been used to chemically modify SPCE. This modified transducer was used as an electrochemical platform to develop an impedimetric genosensor for the detection of transgenic *Bt* maize. The NH_2 -ssDNA probe was covalently immobilized onto COOH-MWCNT-SPCE using carbodiimide chemistry and the impedimetric assessment of the hybridization reaction was carried out using the common redox marker ferricyanide/ferrocyanide $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The change in interfacial charge transfer resistance between the solution and the genosensor was registered and recorded to confirm the hybridization reaction. Noncomplementary DNA sequences were also used to verify the specificity of the methodology (e.g., with a different number of base mismatches) (Bonanni et al., 2009).

An electrochemical impedance sensor for the detection of the transgenic phosphoenolpyruvate carboxylase (*PEP*) gene based on the incorporation of Au-Pt NPs (Au-PtNP) and electropolymerized polytyramine (Pty) onto a GCE. The use of Pty films increased the active electrochemical area, which in turn enhanced the conductive properties of the Au-PtNPs. Several voltammetric techniques were used to evaluate the performance of this genosensor including cyclic voltammetry, SWV, and electrochemical impedance spectroscopy (EIS). Using the electrochemical signal of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair, a dynamic range of *PEP* DNA was observed from 1.0×10^{-12} to 1.0×10^{-7} mol/L with a LOD of 3.6×10^{-13} mol/L (Yang et al., 2012).

Duwensee et al. (2009) described an electrochemical multiplex genosensor for the simultaneous detection (in the same vessel) of four GM DNA sequences from maize.

This assay intended to assess the presence of *SSIb* (starch synthase gene IIb), *ivrp* (invertase gene), *Cry* (the cry-Ia/b transgene), and MON810 (the MON810 specific insertion locus in the maize genome). These specific targets made it possible to detect the presence of maize (*SSIb* and *ivrp*), the existence of transgenic maize (*cry1a/b*), and the presence of a specific event (MON810). In this assay the four different targets were labelled with osmium tetroxide bipyridine [$\text{OsO}_4(\text{bipy})$] to amplify the electrochemical signal produced during the hybridization reaction. The labelled targets were hybridized with complementary DNA immobilized onto AuE and SWV was used to assess this reaction. The hybridization temperature was crucial to obtain optimal electrochemical signals and DNA targets of 25–200 nmol/L were registered.

This multiplex genosensor was also used to detect GM modified maize (MON810) in transgenic flour samples (Mix et al., 2012). The four DNA targets were extracted from the GM samples and amplified using an asymmetric PCR. The transgenic DNA samples were tagged with [$\text{OsO}_4(\text{bipy})$] and hybridization was performed between the labelled targets and the immobilized ssDNA capture probes on the AuE. The hybridization temperature was found to be even more crucial for real samples than the synthetic samples. The authors also verified that the threshold of 0.9% of GM products could be monitored after 4–8 min of hybridization, although 30 min was necessary to detect GMO levels of less than 0.9% of transgenic DNA (Mix et al., 2012).

Sun et al. (2013, 2014) developed an electrochemical reduced graphene (ERG)-modified carbon ionic liquid electrode (CILE) for the detection of transgenic maize (MON810) and soybean (A2704-12). The CILE was constructed and used as a working electrode with 1-butylpyridinium hexafluorophosphate as a binder. ERG was deposited onto the CILE surface, which increased the surface area and led to an increase in the adsorption of ssDNA. ssDNA capture probes were immobilized onto the modified CILE surface via covalent linkage between the unreduced oxygen groups in the ERG and the NH_2 group at the 5' end of the ssDNA. The reduction peak current of the electroactive methylene blue was used as the electrochemical indicator of DNA hybridization and DPV was used to monitor the hybridization. A direct relationship between the concentration of ssDNA was observed in the 1.0×10^{-11} to 1.0×10^{-6} mol/L range for MON810 with a LOD of 4.52×10^{-12} mol/L. A similar relationship was seen for A2704-12 between 1.0×10^{-12} to 1.0×10^{-6} mol/L with a LOD of 2.9×10^{-13} mol/L. The inherent advantages, such as simplicity, efficiency, cost-effectiveness, and environmental friendliness meant that genosensors were applied for the detection of PCR products in transgenic maize and soybean with satisfactory results (Sun et al., 2013, 2014).

Lien et al. (2010) developed a label-free genosensor for the detection of *P35S* using MWCNTs doped polypyrrole and QCM and EIS techniques. MWCNTs were functionalized (with COOH groups) by oxidation, which involved ultrasonic treatment in a mixture of

concentrated $\text{H}_2\text{SO}_4/\text{HNO}_3$. The ends and sidewalls of the treated MWCNTs were then decorated with carboxyl groups. This form of functionalization produced good solubility in an aqueous medium and retained the primary electronic and mechanical properties (Lien et al., 2010). For the electrosynthesis of polymeric films, pure polypyrrole was electrochemically deposited onto Au microelectrodes by applying a constant potential of 0.7 V for 30 min. After this the MWCNTs were added to the Au microelectrodes. Hybridization was followed by QCM and EIS and both techniques, showed that the MWCNTs-PPy films were more sensitive than the bare Au microelectrodes.

Biomolecular analytical systems using computing functionality can be used to mimic the generation process of an event-specific gene and its potential use in the development of an electrochemical genosensor with the ability to detect multiple GMOs. This novel approach provides an alternative to the multiplex analyses in an array format (Liao et al., 2013).

The concept of biomolecule-processed computational screening of multiple ssDNA fragments serves as the core function of a GMO analytical platform that circumvents the need for stepwise analysis of promoter and coding fragments. This logical approach of analyzing GMOs offers a decisive answer on the basis of molecular computations and thus excludes personal errors (Kim et al., 2010). This approach provides an alternative to the complicated protocols involved in the screening of multiple genes with subsequent digital interpretation (Liao et al., 2013).

A multichannel, electrochemical potentiostat was used for the development of multiplex genosensors. Multiplex genosensors were performed on 16-sensor AuE array chips and several transgenic DNA target sequences were used in these experiments including screening sequences *35S* and *G1B1* (promoters); transgenic genes *CP4-EPSPS*, *pat*, and *cordaA*; endogenous genes *lectin* (soybean) and *SSIb* (maize); and the event-specific *GTS 40-3-2*, *A5547-127*, *NK603*, *T25*, and *LY038*. SH-ssDNA capture probes were self-assembled onto AuE array chips by chemisorption. A cascade of two enzymes [HRP-conjugated FITC antibody (α -FITC-HRP) and avidin-conjugated glucose oxidase (Av-GOx)] were used to amplify the electrochemical signal. Electrochemical detection of hybridization was performed using chronoamperometry by incubating TMB and glucose (electroactive substrates) on AuE chip arrays for 2 min. Electrochemical measurements were performed at -100 mV for 60 s. This novel bioanalytical system discriminated various concentrations (0–20 nmol/L) and percentages (0%–5%) of GM genes and material and produced a LOD of 225 pmol/L (Liao et al., 2013).

SPR is a powerful optical technique that can detect and quantify biomolecular interactions that occur between a thin gold-coated prism and an analyte. The principle of this technique is based on the determination of refractive index changes at the interface between materials with different refraction indices (e.g., the surface of the prism covered with a thin metal layer). When biomolecules are immobilized onto the surface sensitive changes occur in the

reflectivity. These changes indicate the extent to which biomolecule binding has occurred (Arugula et al., 2014; Campbell and Gibum Kim, 2007).

Mariotti et al. (2002) developed a SPR-based genosensor for screening analyses (*P35S* and *TNOS*) of GMOs. ssDNA probes (from *P35S* and *TNOS*) were immobilized on a 11-mercaptopundecanol and carboxylated dextran-modified Au chip and hybridization reactions between the immobilized and complementary DNA were performed. Calibration curves were linear at 1–100 nmol/L for *TNOS* and 1–125 nmol/L for *P35S* and both targets showed a LOD of 1 nmol/L. SPR was used to quantify CRM containing 2% transgenic soya flour. For this purpose transgenic DNA was extracted from the CRM and amplified by PCR. Amplified DNA was subsequently denatured with alkaline agents, separated with MNP, and successfully recognized by the immobilized DNA.

Feriotto et al. (2002) developed genosensors for the detection of lectin and Roundup Ready (RR) soybean genes using a biospecific interaction analysis. Different length biotinylated ssDNA fragments from lectin and RR (13-mer for lectin and 15-mer for RR) were immobilized onto SPR Au sensor chips. Label-free hybridization reactions using increasing concentrations of synthetic targets and amplified asymmetric PCR products were then performed. The authors observed that SPR-based genosensors were useful for the real time assessment of PCR products in transgenic soybeans.

Optical silicon thin-film biosensor chips were developed for the simultaneous detection of six GM maize lines (*Bt11*, *Bt176*, GA21, MON810, NK603, and T25) (Bai et al., 2010). ssDNA sequence probes were modified with aldehyde functional groups, which were subsequently arrayed and covalently attached to the surfaces of hydrazine-derivatized chips. Biotinylated PCR amplicons were then hybridized with the immobilized ssDNA probes. Finally, multiplex genosensors were incubated with HRP-conjugated anti-biotin IgG and a precipitable HRP substrate. Hybridization produced an enzymatic reaction between HRP and the substrate, which was visualized by a color change from gold to blue (or purple) on the chip surface. The multiplex genosensor was considered to be extremely robust, highly sensitivity, specific, and it detected all GMOs on the chip (Bai et al., 2010).

Zhu et al. proposed an electrochemiluminescent genosensor for detecting transgenic *P35S*. Their method consisted of immobilizing biotinylated ssDNA probes on streptavidin-coated magnetic beads upon a Pt working electrode by activating the magnetic field. Sandwich hybridization was carried out by performing an initial hybridization step between the target DNA and the biotin probe and a second hybridization step between ruthenium(II) tris-bipyridine (TBR) ssDNA probe and the target DNA. The TBR-hybrid had the ability to react with tripropylamine to emit light, which was detected by electrochemiluminescence. Target DNA levels were directly proportional to the electrochemiluminescent emission of the TBR. Calibration curves were plotted and showed linearity from 5 nmol/L to 5 μ mol/L of *P35S* DNA and a sensitivity of 5 nmol/L. This assay has been used to discriminate transgenic and

nontransgenic tobacco samples. This sensor allowed the rapid, simple, inexpensive, safe, sensitive, and reliable assessment of GMOs in real samples (Zhu et al., 2010).

QCM is a mass sensitive piezoelectric device based on an oscillatory quartz crystal that is capable of detecting mass (e.g., ng) changes. Therefore, QCM detects hybridization reactions that produce increases in the mass and decreases in the resonance frequency. In this sense, piezoelectric genosensors for GMO assessment are based on detecting an increase in mass due to hybridization between the ssDNA capture probe and the label-free transgenic DNA target (Arugula et al., 2014).

Passamano and Pighini (2006) aimed to develop a QCM genosensor for the detection and quantification of MON810. The principles of this genosensor were based on the direct immobilization of MON810 biotin–ssDNA probes onto streptavidin-coated gold crystal surfaces. Label-free hybridization between MON810 and the immobilized ssDNA probes was accomplished using resonance frequency changes. This QCM genosensor was successfully employed for the detection of MON810 in GM maize flour and a cookie made with the GM flour. Linearity between the resonance frequency and the amount of GM was found in the 0.1%–5% range of GM MON810 maize flour.

Stobiecka et al. another QCM genosensor was developed for the detection of transgenic RR soybean. The genosensor was constructed by immobilizing 21-mer ssDNA from the *EPSPS* gene onto Au piezoelectrodes using avidin–biotin interactions. Hybridization reactions were studied between the immobilized ssDNA probe and the synthetic transgenic DNA target or DNA extracted from animal feed containing 30% RR (PCR-amplified and nonamplified). This genosensor presented a LOD of 4.7×10^5 numbers of genome copies contained the RR gene. The major advantage of this genosensor was the direct determination of GMOs in real samples that were not amplified by PCR (Stobiecka et al., 2007).

Minunni et al. developed a similar QCM genosensor to target *P35S* and *TNOS* in CRM soybean powder containing 2% transgenic soybean. Although this system was very specific the transgenic detection concentration (2%) was higher than the limits (0.9%) established by the EU (Minunni et al., 2001).

Karamollaoğlu et al. (2009) developed a mass sensitive QCM genosensor for *P35S* detection using a different immobilization procedure. In this study, immobilization of SH–ssDNA probes was performed by chemisorption onto Au surfaces through the formation of a SAM (thiol–gold interaction). This thiolated DNA sensor was used to trace *P35S* from tobacco plants. DNA from real samples obtained from PCR amplification, digestion fragmentation, and ultrasonication were tested with this QCM sensor. The authors concluded that the genosensor had the ability to detect amplified and nonamplified DNA and therefore represented a promising tool for the real-time, label-free, and direct detection of transgenic DNA (Karamollaoğlu et al., 2009).

4 Conclusions

Developments in biotechnology have modified agriculture through the introduction of GMOs with interesting agronomic characteristics. The rapid advance in the cultivation of GMOs has presented significant impacts in terms of socioeconomic, environmental, and human health benefits; however, despite the advantages offered by GMOs, they are still not globally accepted. Considering these global preoccupations some governmental regulatory agencies have established compulsory labelling rules.

To guarantee that these regulations are monitored, the implementation of appropriate, accurate, and reliable methods are needed. The best analytical tools target the proteins encoded by the inserted gene or the DNA sequences inserted onto the plant genome.

Several protein- and DNA-based techniques have been implemented for the detection and quantification of GMOs; however, conventional methodologies (i.e., ELISAs and PCR), which are used in routine protocols, are laborious, expensive, and time-consuming. Biosensors appear to be appropriate bioanalytical tools for the simple, portable, and low-cost assessment of GMOs. Immunosensors and genosensors seem to be good strategies for transgenic analysis; however, genosensors are ideal for the unequivocal identification of GMOs.

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Further Reading

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Genetically Engineered Food Crops to Abiotic Stress Tolerance

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1 Introduction

Plant foods have been the main source of nutrients and energy for the human population; however, the global population is rapidly multiplying and thus a significant increase in agricultural productivity is required. The growth and development of crops can be affected by abiotic stresses, such as drought, salinity, and temperature (Ahmed et al., 2013; Hu and Xiong, 2013; Mittler and Blumwald, 2010; You and Chan, 2015). In addition, global climate changes are also compromising food security in the world. Therefore, the development and utilization of stress-resistant crops is an efficient strategy to minimize yield losses caused by adverse conditions.

Understanding the mechanisms through which plants perceive and transduce stress signals is essential for the engineering of stress-tolerant crops (Sanchez et al., 2011). Molecular and biochemical studies demonstrate the involvement of a signal transduction cascade in response to abiotic stresses in plants (Hu et al., 2012).

The most practical definition of a biological stress is an adverse effect or a condition that inhibits the normal operation and well-being of a biological system; however, in biological terms it is difficult to accurately measure the force exerted by stress and therefore it becomes difficult to define this term. Under biological conditions a stress may cause injury to one plant but be beneficial to another. Some cultures with stress-specific phenotypes serve as the ideal starting material for the isolation and characterization of stress-associated genes (i.e., those involved in osmotic-stress resistance). These cultures are well suited to harsh environments and they can thrive in conditions of rain, drought, or low fertility soils and they have high productivities (Heidarvand and Maali Amiri, 2010; Sanchez et al., 2011). However, it is necessary to differentiate drought tolerance from desiccation tolerance. Both have basic common features but desiccation tolerance refers to an extreme situation defined by survival

when the water content is less than 5% (0.05 g H₂O/g dry weight). Organisms with this feature are said to be anhydrobiotic (Hoekstra et al., 2001).

In plants there are three physiological responses to restore turgor: (1) the induction of genetic programs hormonally mediated through abscisic acid (ABA), (2) the long-term accumulation of protective molecules, and (3) the closure of stomata of the leaf (Hirayama and Shinozaki, 2010; Tardieu et al., 2014).

Cold, salinization, and heat cause different effects in plants. They all trigger a series of morphological, physiological, biochemical, and molecular changes that adversely affect the growth and productivity of economically important crops. Some of these changes include the inhibition of shoot growth and increased root growth, adjustment in ion transport (Hirayama and Shinozaki, 2010; Tardieu et al., 2014), disruption of water and ionic balance, increased production of toxic metabolic (i.e., reactive oxygen species, ROS), disorganization of membranes (i.e., phospholipid hydrolysis), inhibition of photosynthesis, altered nutrient acquisition (You and Chan, 2015), and alteration in the regulation of osmotic-stress gene expression (Wan et al., 2011).

Therefore, a search was initiated to identify plants that were resistant to pests and diseases, had enhanced nutritional value, and could make an important contribution to food production (Heidarvand and Maali Amiri, 2010; Sanchez et al., 2011). However, generating these types of plants through classical breeding techniques has limitations as the introduction of a gene (or set of genes) requires repeated crossing and selection cycles, making this process restricted to species with sexual reproduction. In addition, genes with undesirable characteristics are also transferred with the target genes during the process (Wang et al., 2016).

The advent of modern biotechnological tools (i.e., recombinant DNA technology) has provided an effective alternative to conventional techniques, as biotechnology allows the genetic material of plants to be altered using genes from any other living being. It is even possible to exchange genetic material between sexually incompatible species (Wang et al., 2016).

Identifying genes conferring advantageous agronomic traits is essential for biotechnology breeding programs. Genes encoding proteins with proven features, such as resistance to biotic (i.e., pests and diseases) and abiotic stresses (i.e., drought and temperature extremes), have been successfully used in the production of genetically modified plants (Heidarvand and Maali Amiri, 2010; Sanchez et al., 2011; Wang et al., 2016).

Plants have evolved several signal transduction pathways that regulate stress-response genes (i.e., kinases, transcription factors, and molecular chaperones), which give insights into how environmental factors are tolerated (Wang et al., 2016; You and Chan, 2015). It is hard to imagine that a single gene can confer drought tolerance since this trait is known to be

determined by multiple genes (Atkinson and Urwin, 2012; Mittler and Blumwald, 2010; You and Chan, 2015); however, despite our limited knowledge of the underlying metabolism of stressed cells, there are known transgenes encoding single transcription factors that confer resistance to drought (Hirayama and Shinozaki, 2010; Pandey and Shukla, 2015).

The phenomenon of tolerance or susceptibility to abiotic stress is very complex, in part because stress can occur at various stages of plant development and because more than one stress can simultaneously affect the plant (Atkinson and Urwin, 2012; You and Chan, 2015). Three interconnected aspects of the plant are important to achieve tolerance: (1) damage should be avoided or minimized and detoxification of harmful elements should occur, (2) homeostatic conditions in the new stressed environment must be recovered, and (3) the plant should resume growth, albeit at a reduced rate (Wang et al., 2016). Some of the more important physiological, biochemical, and molecular processes involved in the plant response to environmental stress are presented in Fig.10.1.

Physiological responses of plants under abiotic stress include a change in the stomata regulation and a reduction of photosynthesis. Physiological responses to drought, which limits the plant growth and crop productivity, include the osmotic stress-imposed constraints on plant processes and interference on nutrient acquisition as the soil dries (Huang et al., 2012). At the biochemical and molecular level, the production of secondary metabolic products (ROS) is increased and several regulatory molecules and genes are activated. This results in the stimulation of enzymes

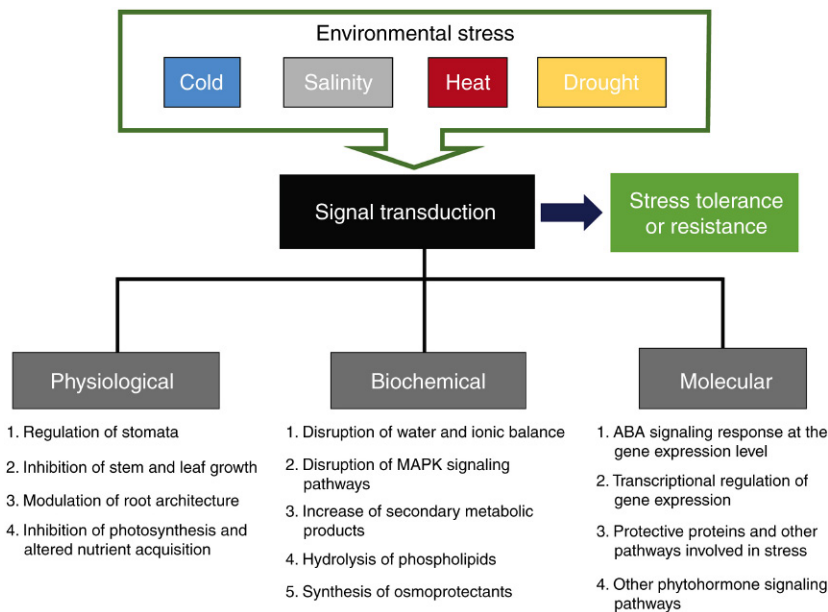


Figure 10.1: Physiological, Biochemical, and Molecular Processes Activated by Environmental Stresses in Plants.

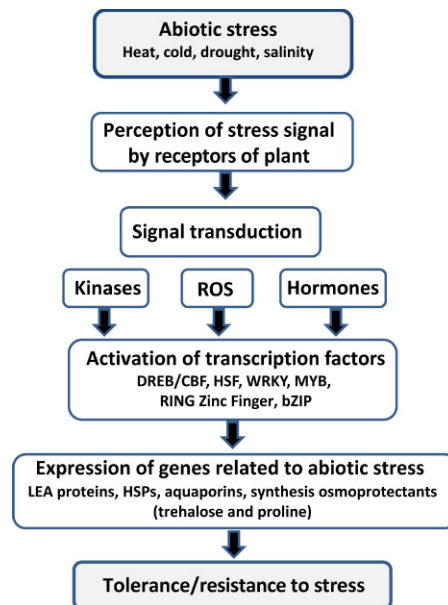


Figure 10.2: Plant Response due to Abiotic Stress With the Activation of Transcription Factors That Regulate the Expression of Stress-Responsive Genes, Culminating in the Resitance/ Tolerance of Plant to Stress.

bZIP, Basic region/leucine zipper; *DREB*, dehydration-responsive element-binding; *HSF*, heat stress transcription factor; *HSP*, heat shock proteins; *ROS*, reactive oxygen species.

involved in the synthesis of osmoprotectants and hormones, as well as the increased production of stress-protectant proteins.

As shown in [Fig. 10.2](#) the stress signals are first detected by plant receptors. Through the activation of secondary messengers this initiates an intracellular signaling cascade, activating transcription factors that induce the expression of genes involved in cell protection ([Atkinson and Urwin, 2012](#); [Hirayama and Shinozaki, 2010](#); [Huang et al., 2012](#); [You and Chan, 2015](#)). Examples of molecules with protective roles against abiotic stress include heat shock proteins (HSPs); late embryogenesis abundant (LEA) proteins, which acts as molecular chaperones by controlling the folding and conformation of other proteins ([Kovacs et al., 2008](#); [Wang et al., 2004](#)); trehalose, which acts as an osmoprotectant and stabilizes biological structures during abiotic stress ([O'Hara et al., 2013](#)); and proline, which acts as an osmoprotectant to stabilizes proteins and protect against denaturation ([Lv et al., 2011](#); [Moustakas et al., 2011](#)). More information about these protective molecules will be presented in the following sections.

Molecular studies have shown that several proteins play different roles in stress responses. Transcription factors, kinases, and phosphatases are involved in the regulation of stress-inducible genes and in the perception of stress and signaling ([Atkinson and Urwin, 2012](#); [Hirayama and Shinozaki, 2010](#); [Pandey and Shukla, 2015](#)).

The two main signaling processes activated under cold and drought conditions are:

(1) osmotic stress, using the mitogen-activated protein kinase (MAPK) module (Sah et al., 2016), and (2) transcription factor regulation at the transcriptional level [i.e., the activation of genes mediated by ABA (Hirayama and Shinozaki, 2010)] modulating the physiological and metabolic responses of the plant (Mizoi et al., 2011; Wang et al., 2016).

The first signaling process may contribute to the production of compatible osmolytes and antioxidants. It may also regulate the cell cycle under osmotic stress through MAPK modules, which are involved in growth regulation, development, cell death, and response to a variety of environmental stimuli (i.e., temperature, drought, ROS, and UV light) (Sah et al., 2016).

The second signaling process can be modulated by the ABA-responsive element (ABRE) binding factor (ABF) protein. The ABF is the major *trans*-factor that binds to the active *cis*-element ABRE (Wang et al., 2016). However, not all drought responses are mediated by ABA, some occur independently through apetala2/ethylene response element-binding factor (AP2/ERF) members (e.g., WRKY, bZIP, MYB, MYC, or NAC) (Sah et al., 2016; Wang et al., 2016; You and Chan, 2015). Transcription factors belonging to the AP2/ERF family indicate that the ABA-independent pathway of drought response should be related to ethylene, since ERF silencing produced plants with a higher drought tolerance (Sharoni et al., 2011; Wan et al., 2011).

Genes encoding molecules involved in plant protection against abiotic stress have been used to produce transgenic food crops with stress tolerance traits. Some examples are presented in Table 10.1. One important aspect in the production of transgenic plants is the genetic transformation method. Although the genetic transformation of plants is routine for many species, the efficiency may be low for others, and some may not have well-established transformation protocols. Thus studies related to gene function are still limited to certain species and must be performed in model plants whenever possible. Model plants, such as tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* are easily processed, have short life cycles, and produce many seeds.

The following sections provide information on the genetic transformation methods widely used in the generation of transgenic plants, including abiotic stress-tolerant food crops.

2 Genetic Transformation of Plants

Besides the isolation and characterization of genes involved in the abiotic stress response, genetic transformation of plants is also an important molecular approach for understanding the roles of genes and their products in tolerance, behavior, and plant phenotypes in the field. This approach is responsible for the generation of genetically engineered plants and it allows researchers to elucidate the effects of overexpression and silencing. This is

Table 10.1: Food crops genetically engineered for abiotic stress tolerance.

Genes	Transgenic Crops	Phenotypic Effects	References
<i>HVA1</i> (LEA protein)	Rice (<i>O. sativa</i>)	Increased tolerance to drought and salinity	Xu et al. (1996)
<i>AtNHX1</i> (Na ⁺ /H ⁺ antiporter)	Tomato (<i>Lycopersium esculentum</i>)	Enhanced tolerance to salinity	Zhang and Blumwald (2001)
<i>HVA1</i> (LEA protein)	Mulberry (<i>Morus alba</i>)	Enhanced tolerance to drought, salinity, and cold	Checker et al. (2012)
<i>TPSP</i> (trehalose synthesis)	Tomato (<i>L. esculentum</i>)	Enhanced drought and salt tolerance	Lyu et al. (2013)
<i>lbOr</i> (orange gene related to carotenoid synthesis)	Sweet potato (<i>Ipomoea batatas</i>)	Salt-stress tolerance	Kim et al. (2013)
<i>LEA Rab28</i> (LEA protein)	Maize (<i>Z. mays</i>)	Enhanced drought stress tolerance	Amara et al. (2013)
<i>P5CS gene</i> (proline synthesis)	Pigeon pea (<i>Cajanus cajan</i>)	Enhanced salt stress tolerance	Surekha et al. (2014)
<i>AtVP1</i> (Vacuolar H ⁺ -pyrophosphatase gene)	Barley (<i>H. vulgare</i>)	Increased tolerance to salinity	Schilling et al. (2014)
<i>CaBZ1</i> (bZIP transcription factor)	Potato (<i>Solanum tuberosum</i>)	Enhanced drought tolerance	Moon et al. (2015)
<i>cb11</i> , <i>cb15</i> , and <i>cb110</i> (calcineurin B-like protein)	Chickpea (<i>C. arietinum</i>)	Tolerance to salinity and dehydration	Meena et al. (2015)
<i>AP2/ERF</i> (transcription factor)	Rice (<i>O. sativa</i>)	Drought tolerance	Jisha et al. (2015)

possible by cloning the gene into a vector under the control of a constitutive, inducible, or tissue specific promoter (i.e., the CaMV35S) or using cassettes with gene fragments containing sense and antisense orientations separated by an intron (i.e., for silencing) ([Zalewski et al., 2012](#)).

The integration of the exogenous DNA (transgene) into the plant genome is a crucial step for effective expression and transmission across generations.

2.1 Biolistic-Mediated Genetic Transformation

The biolistic or microparticle bombardment was first reported by [Sanford et al. \(1987\)](#). It is a direct method of gene transfer, which does not require the use of biological vectors, and it has been shown to be effective in studies for drought and salinity tolerance ([Ganguly et al., 2012](#); [Xu et al., 1996](#)). The method is also broadly applicable for a range of food crops including maize ([O'Kennedy et al., 2011](#)), wheat ([Tamás-Nyitrai et al., 2012](#)), and tomatoes ([Ruf and Bock, 2014](#)).

Many models of microparticle bombardment incorporate DNA into the target tissue using a helium pulse at high pressure using a gene gun or a biolistic device. This shoots millions of DNA-coated metal (gold or tungsten) microparticles carrying the gene of interest at the target

cells or tissues. The DNA elutes off particles that become lodged inside the cells and some of this DNA may become stably incorporated in the host genome (Kikkert et al., 2005). Thus the coding sequence of a gene of interest can be integrated into the nucleus after accelerated particles penetrate into the cell.

Special equipment is required for particle bombardment and the development of specific protocols is commonly required for each new genetic transformation experiment (Sanford, 1990). Disadvantages of this approach include the possibility of mechanically shearing large plasmids during particle preparation or delivery, and chemical reactions using tungsten may promote the cleavage or modification of DNA (Venkatesh and Park, 2012).

However, biolistic genetic transformation can be used for both monocots and dicots and it is therefore potentially applicable to a wide variety of cells and tissue (O'Brien and Lummis, 2006). It has also been shown to effectively deliver DNA into plastids in a variety of plant species (Ruf and Bock, 2014; Venkatesh and Park, 2012). Biolistic genetic transformation can be used on large numbers of cells and it is rapid and simple to use (Sanford, 1990).

2.2 *Agrobacterium-Mediated Genetic Transformation*

The so-called indirect genetic transformation methods are those based on the use of a biological vector. Among these methods, transformation mediated by *Agrobacterium* (a widespread naturally occurring soil bacterium) has been widely used for the introduction of exogenous DNA into plant cells and this has been effectively used for the generation of abiotic-stress tolerant plants (Shimosaka and Ozawa, 2015; Xu et al., 2015; Yuan et al., 2015).

Within the *Agrobacterium* genus, five species have the ability to penetrate into the plants (predominantly dicotyledonous but also some monocotyledonous plants). *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* concentrate hundreds of host plant species. *A. tumefaciens* was first used to modify tobacco (Herrera-Estrella, 1983), and it has since become the most studied *Agrobacterium* for the genetic transformation of plants. *A. rhizogenes* can also be used for transformation and it stimulates the proliferation of secondary roots from the infection point.

Plasmids are essential for the genetic transfer of the coding sequence of interest. The plasmid used for *A. tumefaciens* is called tumor inducing (Ti), and the plasmid used for *A. rhizogenes* is called root inducing (Ri). Ti and Ri plasmids have small and mobile segments of transfer DNA (T-DNA) that are transferred to the plant cell nucleus and integrated into the plant genome. T-DNA carries genes coding proteins involved in the biosynthesis of *trans*-zeatin (cytokinins), auxins, and opines (de la Riva et al., 1998).

After transforming an *Agrobacterium* strain with the vector (containing the gene of interest), the bacterium is brought into contact with any explants that have the potential for plant regeneration. Thus the *Agrobacterium* begins infection by transferring DNA and transforming

the host genome. After infection, the entire plant is regenerated, *Agrobacterium* is eliminated, transformed cells are selected, and transformed plants are evaluated.

Advantages of using *Agrobacterium*-mediated transformation in comparison to other methods of transformation include the reduced number of transgene insertions and the intact and stable integration of the transgene into the plant genome. The integration of high numbers of transgene copies often leads to transgene silencing (Jones et al., 2005; Travella et al., 2005).

Agrobacterium have been mostly used for monitoring plant phenotypes during overexpression or silencing of abiotic-stress genes. Many studies have also reported the suitability of *Agrobacterium* over the biolistic method (Shou et al., 2004; Zalewski et al., 2012). For example, Shou et al. (2004) reported that transgenic maize overexpressing the *Nicotiana* protein kinase (*NPK1*) gene showed increased tolerance to drought. *Agrobacterium*-mediated genetic transformation of this crop produced lower transgene copies and higher and more stable gene expression compared to plants generated using biolistic methods. Zalewski et al. (2012) verified that the biolistic transformation of *HvCKX2* in barley showed low productivity and altered plant development in comparison to the *Agrobacterium* method.

The major difference between the two methods is the presence of multiple copies of the transgene (and large rearrangements) in the biolistic method. An epigenetic consequence of the destructive biolistic method (both physically and genetically) may be somaclonal variation (Zalewski et al., 2012).

3 Proteins With Protective Roles Against Abiotic Stress

3.1 Late Embryogenesis Abundant Proteins

The LEA proteins were first discovered in the late developmental stages of plant seed embryos (Galau et al., 1986), and they are among the proteins most widely involved in abiotic-stress responses. Genes encoding LEA proteins are differentially expressed in response to drought (Behringer et al., 2015; Bhardwaj et al., 2015), dehydration (Hand et al., 2011), elevated ABA concentrations (Shinde et al., 2012; Zhao et al., 2011), high salinity (Costa et al., 2011; Pedrosa et al., 2015), extreme temperatures, and oxidative stress (Sharma et al., 2016; Su et al., 2011).

LEA proteins are members of a large group of glycine-rich, low molecular weight (10–30 kDa) proteins (Duan and Cai, 2012). In higher plants they predominantly consist of hydrophilic amino acids ordered in repeated sequence (e.g., Gly and Lys) to generate hyperhydrophilic, thermostable proteins (Hong-Bo et al., 2005). They are considered to act as hydration buffers (Garay-Arroyo et al., 2000), molecular chaperones (Kovacs et al., 2008; Liu et al., 2010), cell membrane protectants (Babu et al., 2004), ROS sequestrants (Hara et al., 2004), and act against protein aggregation (Boucher et al., 2010; Nakayama et al., 2008).

Table 10.2: Subcellular distribution of *A. thaliana* LEA proteins encoded by 51 genes clustered in nine families.

LEA Family	Subcellular Location
LEA-1 LEA-2 LEA-5 LEA-6 Dehydrin SMP	Cytosolic (or cytosolic and nuclear)
AtM	Secretory pathways
LEA-3	Mitochondrial or cytosolic
LEA-4	Multiple locations (cytosol, plastid, mitochondria, endoplasmic reticulum, and pexophagosome)

LEA, Late embryogenesis abundant; SMP, seed maturation proteins.

Source: Data obtained from Candat, A., Paszkiewicz, G., Neveu, M., Gautier, R., Logan, D.C., Avelange-Macherel, M.H., Macherel, D., 2014. The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in *Arabidopsis* offers tailored protection against abiotic stress. *Plant Cell* 26, 3148–3166.

Most LEA proteins are cytoplasmic and hydrophilic (Battaglia et al., 2008); however, some atypical LEA proteins are hydrophobic (He et al., 2012a). LEA proteins may also be classed as intrinsically disordered proteins (Candat et al., 2014; Chakrabortee et al., 2012) and they are divided based on the motifs present in the amino acid sequence of each protein (Battaglia et al., 2008; Dure et al., 1989).

In the PFAM database, proteins encoded by LEA genes in *A. thaliana* are clustered into eight families based on their primary sequence: dehydrin, LEA-1, LEA-2, LEA-3, LEA-4, LEA-5, LEA-6, and seed maturation proteins (SMP); proteins without PFAM classification are in a group referred to as AtM (Candat et al., 2014). The subcellular distribution of these nine families is summarized in Table 10.2. Candat et al. (2014) suggested that the broad subcellular distribution of LEA proteins highlights the requirement for each cellular compartment to be provided with protective mechanisms to cope with desiccation or cold stress.

Studies of the heterologous expression of LEA proteins in *Escherichia coli* demonstrated that these proteins play an important roles in tolerance to cold temperatures (He et al., 2012a; Hu et al., 2012; Liu et al., 2015), osmotic stress (He et al., 2012a), heat (Barros et al., 2015; Gao and Lan, 2016; He et al., 2012a), ultraviolet radiation (He et al., 2012a; Hu et al., 2012), drought (He et al., 2012a; Wu et al., 2014), and high salinity (Barros et al., 2015; He et al., 2012a; Wu et al., 2014). The relationship between LEA protein expression in food crops and the generation of tolerant plants has also been reported.

Duan and Cai (2012) found that transgenic rice plants overexpressing *OsLEA3-2* showed significantly stronger growth performance under salinity or osmotic stress conditions and were able to recover after 20 days of drought stress. Checker et al. (2012) observed that overexpression of *Hva1* (an LEA gene from barley) in mulberries produced berries with

enhanced tolerance against drought, salinity, and cold stress. The authors verified that HVA1 could regulate downstream genes associated with abiotic-stress tolerance. Overexpression of an LEA protein (from maize) in tobacco conferred tolerance to osmotic and oxidative stress (Liu et al., 2013a) and low temperatures (Liu et al., 2014a). Transgenic maize plants overexpressing the LEA *Rab28* gene showed enhanced water stress and under conditions of osmotic stress transgenic seedlings showed increased leaf and root areas, higher relative water content (RWC), and reduced chlorophyll loss in relation to wildtype plants (Amara et al., 2013).

Sasaki et al. (2014) identified a new LEA protein in wheat and established that its overexpression conferred freezing tolerance in nonacclimated plants. Wang et al. (2014) found that overexpression of the *SiLEA14* gene of foxtail millet improved resistance to osmotic and salt stress and contributed to the increase in free proline and soluble sugar content (i.e., metabolites related to defense against water stress), and the reduced water loss.

Sharma et al. (2016) verified that ectopic expression of peanut AdLEA in tobacco produced enhanced tolerance to dehydration, salinity, and oxidative stress compared to wildtype plants. The authors also observed that transgenic tobacco plants maintained better photosynthetic efficiency under drought conditions and that ROS levels were significantly reduced in the leaves and stomatal guard cells.

These results demonstrate that LEA proteins are fundamental for genetically modifying plants against abiotic stresses.

3.2 Heat Shock Proteins

HSPs were initially identified in response to high temperatures in eukaryotes and prokaryotes, but they are involved in the response to various abiotic stresses. HSPs are mainly located in the cytoplasm, although in plants they are also found in the mitochondria, endoplasmic reticulum, nucleus, and chloroplasts (Boston et al., 1996; Park and Seo, 2015).

HSPs act as molecular chaperones in normal cells and under conditions of stress they help to maintain and/or restore protein structure and homeostasis (Timperio et al., 2008). They also help to maintain normal membrane-associated processes during drought (Maqbool et al., 2009; Sato and Yokoya, 2008) and defend against cellular damage caused by the overcrowding of cytoplasmic components during dehydration. Such crowding is related to protein denaturation and membrane fusion (Hoekstra et al., 2001).

HSPs are classified into five major families based on their molecular weight (15–42 kDa): Hsp70 (DnaK), chaperonins (GroEL and Hsp60), Hsp90, Hsp100 (Clp), and small Hsp (sHsp) family (Wang et al., 2004).

Differential expression studies and combination stress experiments (i.e., drought and high temperature), have confirmed that HSPs of different molecular weights respond against

different abiotic stresses (Grigorova et al., 2011; He et al., 2012b; Liu et al., 2014b; Reddy et al., 2015; Vásquez-Robinet et al., 2010). Under in vivo conditions, HSP gene expression is strongly correlated with heat stress transcription factors (HSFs), which are cytoplasmically-located (inactive) regulatory proteins that are activated by stress conditions and undergo oligomerization and recompartmentalization to the nucleus (Baniwal et al., 2004). Studies have also reported that HSFs gene expression is critical for plant resistance against abiotic stress (Bechtold et al., 2013; Liu et al., 2013b; Shen et al., 2013).

Overexpression of HSPs in *E. coli* and subsequent purification of the recombinant proteins demonstrated their role in thermal tolerance and their ability to act as molecular chaperones (Mulaudzi-Masuku et al., 2015; Sato et al., 1999; Siddique et al., 2003; Reddy et al., 2010, 2014). In addition, these proteins have also been reported to play an important role against abiotic stresses in transgenic plants.

Overexpression of cytosolic ZmHSP16.9 (from maize) in tobacco conferred tolerance to heat and oxidative stresses by increasing the seed germination rate, root length, and antioxidant enzyme activities (Sun et al., 2012).

Chauhan et al. (2012) observed that constitutive expression of chloroplastic TaHSP26 (from wheat) in *Arabidopsis* plants under sublethal continuous heat conditions increased photosystem II (PSII) thermal tolerance, enhanced the biomass, and increased the active photochemical efficiency. The authors hypothesized that this protein may prevent aggregation and facilitate refolding of PSII components, thereby improving photosynthetic efficiencies.

Xu et al. (2013) verified that overexpression of soybean GmHsp90 in *Arabidopsis* plants generated a differential accumulation of proline and decreased the damage from heat, salt, and osmotic stresses. Growth rates (measured as fresh weight) of the transgenic plants were also significantly different from controls.

The transgenic overexpression of OsHSP18.6 in rice produced crops with increased tolerance to heat, drought, salt, and cold temperatures. Increased activities of catalase and superoxide dismutase were also observed under heat and drought conditions compared with the wildtype plant (Wang et al., 2015). Hoang et al. (2015) observed that the exogenous expression of the *Hsp70* gene significantly improved salinity tolerance in transgenic rice at the whole plant level.

Transgenic sugarcane plants overexpressing EaHSP70 showed enhanced tolerance to water deficiency and high salt concentrations, cell membrane thermostability, enhanced RWC and chlorophyll content, and enhanced photosynthetic efficiency. Abiotic stress-responsive genes (i.e. *DREB2*, DNA helicase 45, *LEA*, *RD29*, *ERD*, *ERF*, *Cor15*, and *BRICK*) were upregulated more than 100-fold in each transgenic event compared to the control plants (Augustine et al., 2015).

These results confirm the potential of HSPs for the genetic engineering of food crops with abiotic stress tolerance.

3.3 Aquaporins

Terrestrial plants have to devise strategies to maintain homeostasis when they come into contact with dry air, high temperature, and wind, as these conditions intensify the transpiration rate. Plants are just about able to manage this through the regulation of stomata (Li et al., 2014). However, the situation becomes more aggravated under abiotic stress (i.e., high salinity), in which the osmotic pressure increases, water is lost, and root hydraulic conductivity is reduced (Lpr). This unfavorable situation could be reverted through the transgenic expression of water channels (Horie et al., 2011), which transport water to different tissues and ensure the continuity of processes including cell expansion, stomatal movement, and epinasty (Chaumont and Tyerman, 2014).

Aquaporins (AQPs) are transmembrane proteins that serve as channels for the movement of water and other molecules, such as glycerol, urea, ammonia, hydrogen peroxide, and carbon dioxide (Wu and Beitz, 2007). This mechanism is driven by the osmotic or hydrostatic pressure established between both sides of the membrane and requires low activation energy (Madeira et al., 2016). The AQPs belongs to a superfamily of major intrinsic proteins, which are present in all living organisms with the exception of thermophilic Archaea and intracellular bacteria (Abascal et al., 2014). Plant AQPs are further categorized into the following families: NOD26-like intrinsic proteins (Weaver et al., 1991), plasma membrane intrinsic proteins (Kammerloher et al., 1994), tonoplast intrinsic proteins (Karlsson et al., 2000), small basic intrinsic proteins (Chaumont et al., 2001), and X-intrinsic proteins (Ishibashi et al., 2011).

All AQPs retain four monomers that are each able to form the pore unit (Chaumont and Tyerman, 2014). Each AQP consists of six membrane-spanning domains and five connecting loops (A–E). Loops A and D contain conserved hydrophobic amino acids (NPA motifs), which act in the constriction of the central pore to control the movements of protons. These residues are also used to identify these proteins (Maurel et al., 2015).

The dynamic nature of AQPs when faced with unfavorable environmental pressures is emphasized by two systems: (1) stomatal conductance (gs), which refers to the transpiration and the inherent loss of water vapor, and (2) hydraulic conductivity of the vascular system (Lp), which relates to the absorption of water through the roots (Moshelion et al., 2015). Therefore, AQPs functions to increase the CO₂ transportation in leaves, by PIP1 of *A. thaliana* (Uehlein et al., 2012) and increase the liposomal permeability to water, which is promoted by the same protein in *Oryza sativa* (Scalera et al., 2014). *AtPIP2;2* knockouts exhibited negative changes in the hydrostatic transport of water compared with wildtype (Da Ines et al., 2010). Conversely, the overexpression of *OsPIP1;1* attested the possible involvement of the protein in the acquisition of salt-stress tolerance (Liu et al., 2013). It is relevant to emphasize that AQPs also stimulate morphological changes in answer to stress, as substantiated in *Ocimum basilicum* L. and strawberry (*Fragaria x ananassa* Duch.), where a reduction in stomata density was observed under high salinity conditions (Orsini et al., 2013).

Three cultivars of *Brassica oleracea* var. *italica* (Parthenon, Naxos, and Chronos) demonstrated negative changes in the stomatal conductance, transpiration rate, and turgor potential when treated with NaCl (Muries et al., 2013). PIP2 protein levels were also increased under salt stress, and the same effect was described on PIP1 levels in *Malus prunifolia* (Liu et al., 2012). Furthermore, overexpression of *PIP2;7* in the seedlings of *Gossypium hirsutum* resulted in the increased production of GUS in conditions of drought, suggesting that the promoter of these genes is induced by this stress (Zhang et al., 2012). The regulation of AQP genes by abiotic stresses was proved in *Thellungiella salsuginea*, sweet orange (*Citrus sinensis* L. Osb.), and barley (*Hordeum vulgare* L.) (Hove et al., 2015).

Some studies that aim to expand the biotechnological applications of AQPs for the improvement of food crops are being encouraged (i.e., the use of carbon nanotubes). In these studies the capacity of nanotubes to penetrate the cellular wall or plasmatic membrane (Zhang et al., 2008) enables interference with the AQP transcripts produced in the organism (as observed in tomatoes) (Khodakovskaya et al., 2011). In addition, the overexpression of nonendogenous AQPs can enhance tolerance through combination with the endogenous proteins (Ayadi et al., 2011). Overexpression of AQP genes in *Glycine max* is now producing field data that confirms that they are viable for these stresses. It is therefore justifiable that genetic engineering of this protein family may help the improvement of relevant agronomic crops (Zhou et al., 2014).

4 Signaling Molecules\Transcription Factors

4.1 Calcineurin B-Like Proteins

The plants initially detect stimulation (i.e., high salinity, low temperature, and drought) through membrane receptors. These receptors decode the message before transmitting the signal to intracellular compartments where it stimulates enzymatic reactions that trigger the response (Sánchez-Barrena et al., 2013). Thus the stimulus perception and the answers generated after the signal transduction are vital in adapting to adverse conditions (i.e., salinity), which can result in impairment of nutrient acquisition, accumulation of ROS, membrane disorganization, and photosynthesis repression (Hasegawa et al., 2000).

Calcium ions act as indicators of environmental changes, such that temporal and spatial changes in its cytosolic levels (calcium signatures) induce calcium-binding protein sensors (Yu et al., 2014). These can act in two ways: (1) modulating their own enzymatic activity via conformational changes (i.e., sensor responders), or (2) regulating the activity of other target proteins, such as kinases and transcription factors (i.e., sensor relays) (Hassan et al., 2012).

Calcineurin B-like proteins (CBLs) are named in reference to the phosphatase calcineurin in animals (Kudla et al., 1999). They are sensor relays exclusive to plants (Shi et al., 1999) that

principally interact with and activate CBL-interacting protein kinases (CIPK), permitting their phosphorylation of other proteins (Farani et al., 2015). The first CBL-CIPK network identified was the salt overly sensitive (SOS) pathway, in which SOS3 (CBL4) proteins interact with SOS2 (CIPK24) proteins to form a complex that promotes salinity tolerance (Qiu et al., 2002).

The high calcium affinity attributed to the CBL proteins is due to the four EF-hand domains they all contain, each of which is formed by two α -helices connected by one loop of 12 amino acids. This arrangement allows the coupling of up to four calcium ions (Meena et al., 2015). CBLs can be classified based on their number of canonical sites (Batistic and Kudla, 2009) or the number of amino acids in the N-terminal domain in *Arabidopsis* (Batistic et al., 2008).

CBL proteins are able to regulate ionic homeostasis by collaborating ion uptake and root exclusion, where the gene expression tends to prevail (Xu et al., 2006). Thus soybean (*G. max*) CBL1 increased the tolerance to drought and salt imbalance in *Arabidopsis* (Li et al., 2012). Chickpea (*Cicer arietinum* L.) seed genes (*cbl1*, *cbl5*, and *cbl10*) were upregulated during treatment with NaCl and polyethylene glycol, suggesting that they participate in the acquisition of salt and dehydration tolerance (Meena et al., 2015). From the same point of view, *cbl2* and *cbl3* of vacuolar membrane determined the activity of V-ATPase, interfering with the ionic tolerance and micronutrient acquisition in *Arabidopsis* (Tang et al., 2012). Huang et al. (2011) confirmed the interaction between CBL1 and CIPK7 and the induction of this complex under low temperatures.

CBLs regulate subunits of serine/threonine proteins, CIPKs (Luan, 2009). CBL1 has been reported to interact with CIPK24, CIPK25, and CIPK26 to ensure the ionic homeostasis of Na^+ and K^+ in *Populus euphratica* (Zhang et al., 2013). The protein complex tAtCBL1/9–AtCIPK23 has also been reported to control the attainment of nitrate and K^+ in plants (Hashimoto and Kudla, 2011). Under high concentration of mannitol, NaCl, and ABA and low concentrations of phosphate, *Brassica napus* produced higher levels of CBL1–CIPK6, resulting in enhanced tolerance to adverse conditions compared with the wildtype (Chen et al., 2012a).

Ca^{2+} represents just one method of intracellular communication and requires a higher understanding of the biochemical properties of these sensing proteins. Thus protein characterization can clarify mechanisms of regulation in vivo (DeFalco et al., 2010). As the CBL–CIPK complex increases the versatility of responses to the calcium signatures, analysis of gene expression suggests critical guidelines for manipulating different phenotypes in culture (Cramer et al., 2011). The multiplicity of substrates, with which CBL proteins can act means that it is likely to be related to several physiological plant processes (Kim, 2013). Therefore, understanding how it acts results in knowledge of how plants acclimatize and consequently allows greater efficiency in the improvement of cultures faced with external perturbations (Mittler and Blumwald, 2010).

4.2 Basic Leucine Zipper Proteins

Plants are under a continuous gradient ranging from optimum to adverse conditions. Therefore, multiple responses (i.e., those mediated by transcription factors) have been evolutionarily selected to allow plants to adapt to various scenarios (Llorca et al., 2014). Stresses, such as high soil salinity can have a severe impact on the irrigation systems of agronomically important crops (Munns and Tester, 2008) resulting in socioeconomic and environmental damages. Therefore, efforts aimed at increasing crop tolerance by regulating the expression of stress-related genes are vital (Xu et al., 2016).

The genes that assist in the attenuation of environmental disturbances can be divided into two groups: (1) those that encode regulatory proteins, such as kinases or transcription factors, and (2) those that are involved in cell metabolism or signal-related proteins (Hirayama and Shinozaki, 2010). Transcription factors interact with DNA and other elements within the transcriptional process to regulate the spatial access of RNA polymerase in *cis*-acting elements of the promoter (Udvardi et al., 2007).

The basic region/leucine zipper (bZIP) family is so-called because proteins within this family contain this bZIP domain. These are structurally organized in an α -helix with a conserved basic DNA binding region and a positively charged and a hydrophobic heptad repeat (C-terminal leucine zipper motif, which ensures dimerization and is not conserved) (Banerjee and Roychoudhury, 2017; Ellenberger et al., 1992). Classification within the family is based on sequence similarities and functionalities (Wei et al., 2012).

When tobacco was engineered to overexpress a bZIP ABA-responsive gene from *Poncirus trifoliata* (*abf*) it showed enhanced tolerance to dehydration and drought and lower concentrations of ROS (Huang et al., 2010). The same findings were observed when tobacco plants were engineered to overexpress bZIPs from *Medicago sativa* (Li et al., 2013a) and *Tamarix hispida*. In the later, parameters, such as the amount of chlorophyll suggested salt-stress tolerance (Wang et al., 2010).

Many studies have been performed using the genus *Arabidopsis*, as it is known to contain ~75 bZIPs (Jakoby et al., 2002). Zhong et al. (2015) attested that the genes related to nitrate transport and assimilation were positively regulated by AtTGA4 when it was overexpressed in transgenic lines, giving them an advantage over wildtype lines. Overexpression of corn (*Zea mays*) bZIP72 in *Arabidopsis* produced higher survival rates during drought conditions, increased proline concentration, and enhanced stomatal conductance, indicating a tolerant phenotype (Ying et al., 2012).

The rice genome (*O. sativa*) contains 89 putative bZIPs (Nijhawan et al., 2008). Proteins OsbZIP39, OsbZIP50, and OsbZIP60 are potentially responsible for the perception of ER stress (Takahashi et al., 2012), while OsbZIP50 and OsbZIP39 are regulatory chaperone genes (Lu et al., 2012).

bZIP proteins operate independently and via crosstalk in response to multiple combined stresses. This indicates that plants have developed complex signaling networks as strategies against disturbances (Wang et al., 2016). It is also possible that a limited set of bZIP monomers can combine to enable response plasticity. Therefore, functional analysis of bZIPs can assist in our understanding of the regulatory network (Alves et al., 2013) and in the establishment of crops with resistant phenotypes under real field conditions (Rabara et al., 2014).

4.3 AP2/ERF Proteins

It is generally accepted that APETALA2/ethylene-responsive element binding factor (AP2/ERF) proteins are involved in plant responses to phytohormones, pathogen attack, and environmental stresses (Ma et al., 2010). The AP2/ERF superfamily is a large group of transcription factors characterized by the presence of a highly conserved DNA-binding domain (AP2/ERF-type DNA binding domain). This domain was first found in the *Arabidopsis* homeotic gene, *APETALA2*, and a similar domain was later found in tobacco (*N. tabacum*) ethylene-responsive element binding proteins. Although initially defined as plant specific, the AP2 domain was later found in proteins of protists and cyanobacteria (Licausi et al., 2013; Mizoi et al., 2011; Qi et al., 2011; Sharoni et al., 2011). It consists of ~60–70 residues that confer a typical three-dimensional conformation organized into a layer of three antiparallel beta sheets followed by a parallel α helix (Licausi et al., 2013; Qi et al., 2011).

This superfamily includes four major subfamilies: AP2, related to ABI3/VP1 (RAV), ERF, and dehydration-responsive element-binding (DREB) proteins. Many factors have been reported to be involved in the abiotic stress responses of plants, each with different developmental and physiological functions (Mizoi et al., 2011). A structural analysis of an *Arabidopsis* ERF protein in complex with a target DNA molecule revealed that the AP2/ERF domain contains a three-strand β -sheet in N-terminal, which recognises a target sequence, as well as an α -helix in C-terminal (Mizoi et al., 2011; Qi et al., 2011).

Some authors separate members of the AP2/ERF superfamily into three groups based on the number of DNA binding domains (i.e., AP2 domains) they contain: AP2 subfamily members contain double AP2 domains (tandem); RAV subfamily members contain one AP2 domain associated with a B3 DNA-binding domain; and ERF subfamily and other members contain only a single AP2 domain (Ma et al., 2010; Mizoi et al., 2011). There is a conserved protein called soloist, which contains a single AP2 domain, but its sequence and gene structure strongly diverge from ERF transcription factors. These proteins can be additionally classified as activators or repressors depending on their specific transcriptional activity (Licausi et al., 2013).

DNA binding proteins colonized several eukaryotic genomes and generated new families of transcription factors during evolution. Among the various hypotheses of gene exchange

between plants and other organisms, the lateral transfer of AP2-containing proteins from bacteria (or viruses) to plants is the most likely (Licausi et al., 2013).

Several studies have been performed to assess the influence of AP2/ERF expression on abiotic stress resistance in food plants. It is now known that these proteins have different effects. For examples, analysis of 23 ERF family genes (representing each clade) in tomatoes demonstrated differential expression in response to more than one abiotic stress treatment. This suggests that ERF genes play several roles in plants (Sharma et al., 2016).

These genes may also act in different plant tissues. In *Vitis vinifera*, expression analyses in different aerial tissues showed features previously described in other plant systems, as well as highlighting possible new roles for some ERF members during fruit ripening (Licausi et al., 2010). Available EST information was used as a source of expression data in *Triticum aestivum*, and putative AP2/ERF family genes were detected in nine tissue types. Transcripts were shown to be most abundant in leaves, roots, and seeds, and least abundant in the stem. Most AP2/ERF family members showed some tissue specificity (Zhuang et al., 2011). Temporal expression analysis has also been performed in rice, and genes were mainly found to be expressed in roots, panicles, mature embryos, and seeds (Ma et al., 2010).

In *A. thaliana*, 117 putative AP2/ERF family genes were identified by in silico analysis based on the presence of the conserved AP2/ERF domain sequence (Zhuang et al., 2011). Multiple AP2/ERF genes are present in plants and they are regulated by environmental stresses. For example, 163 AP2/EREBP genes were identified in rice and microarray results suggested that more than 50 of these (nonredundant) genes were upregulated in response to biotic and abiotic stress (Sharoni et al., 2011). Yin et al. (2012) evaluated the response of kiwifruit ERF to low temperature (0°C), high temperature (35°C), high CO₂ (5%), and high water loss (~10% RH air). Expression patterns of 13 ERF genes showed that five were constitutively upregulated, one was generally downregulated, and the others remained unchanged (Yin et al., 2012). Eight genes were differentially expressed during cold or heat stress conditions in Chinese cabbage (Li et al., 2013b).

Members of this family can also influence hormone levels. Gene expression analysis demonstrated that an AP2/ERF protein restricts ethylene-induced enhancement of gibberellin responsiveness during internode elongation by downregulating a gibberellin biosynthetic gene (Qi et al., 2011).

Some genes also have differential expression patterns in different tissues. Chen et al. (2012b) analyzed the expression pattern of AP2/ERF genes in peanuts under cold, salt, and drought stresses. The results indicated that the expression of two genes were rapidly and substantially enhanced under abiotic stress, whereas the expression of another two were slightly enhanced. Some genes were downregulated in leaves experiencing salt and drought stress. Interestingly, the expression of two proteins exhibited contrary expression patterns in peanut leaves and

roots upon PEG treatment. This suggests that different ERF proteins may have different functions in the acclimation of food plants to abiotic stress.

Food and nonfood plants can share high levels of genetic similarity. One study assessed AP2/ERF transcription factors in Chinese cabbage and *Arabidopsis* and established that these genes are distributed among several chromosomes. The sequences of 286 AP2/ERF genes were distributed unevenly among the ten chromosomes of the Chinese cabbage genome, and three genes could not be assigned to any chromosomal location. Gene duplication of AP2/ERF family genes exists on Chinese cabbage chromosomes (Li et al., 2013b).

Hormone treatments also change expression levels of these genes. Real-time quantitative PCR analysis in rice showed that expression levels of the *AP2LP* gene were increased during drought and gibberellin treatments but decreased under low temperature, salt, ABA, and zeatin treatments. These results suggest that this gene may be related to stress responses and it likely plays a role as a transcriptional regulator in the ABA and gibberellin pathways when plants respond to abiotic stresses (Ma et al., 2010).

The expression of some AP2/ERF genes help food plants to simultaneously protect against pathogens and abiotic stresses. Specific interaction between rice and its bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), stimulates a signal transduction pathway of which the rice AP2/ERF transcription factor is a downstream component. Constitutive expression of this gene did not affect normal plant growth; however, microarray analysis revealed that overexpression caused increased expression of lipid metabolism genes (i.e., lipase and chloroplastic lipoxygenase) and genes related to jasmonate and ABA biosynthesis. Comparative analysis of nontransgenic and AP2/ERF overexpressing genotypes revealed that the transcription factor attenuates diseases caused by *Xoo* and confers drought tolerance in transgenic rice. These results suggest that overexpression of the gene activates jasmonate and ABA signaling pathways to enhance survival under abiotic or biotic stress conditions (Jisha et al., 2015).

5 Osmoprotectant Metabolites

5.1 Trehalose-6-Phosphate Synthases (TPS) and Phosphatases (TPP)

Trehalose is a nonreducing disaccharide sugar present in bacteria, fungi, invertebrates, and plants. It is an important mechanistic component of metabolism and plant growth. Its accumulation influences sugar metabolism leading to an osmoprotectant effect under different environmental stresses (Delorge et al., 2014; Nounjan and Theerakulpisut, 2012; Redillas et al., 2012).

The presence of trehalose in several organisms and the existence of multiple trehalose biosynthesis pathways suggest an evolutionary role for trehalose metabolism in nature (Vandesteene et al., 2012; Wahl et al., 2013). Plants only possess one trehalose biosynthetic pathway and this is the pathway that is the most widely distributed in all organisms. It is formed by two consecutive

enzymatic reactions: the first begins when trehalose-6-phosphate synthase (TPS) catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate (G6P) to form trehalose-6-phosphate (T6P) and uridine diphosphate (UDP); the second step involves the dephosphorylation of T6P into trehalose by trehalose-6-phosphate phosphatase (TPP) (Li et al., 2011; Lyu et al., 2013; Redillas et al., 2012; Yadav et al., 2014). As such, TPS and TPP are the major enzymes in trehalose biosynthesis and multiple isoforms of each exist in plant genomes; for example, *A. thaliana*, contains 11 TPS or TPS-like genes and 10 TPP genes (Delorge et al., 2014).

Trehalose was first identified from the ergot fungus of rye (Li et al., 2011). It is considered as an osmoprotectant because it counters the effects of desiccation from drought, salt, or low-temperature in plants (O'Hara et al., 2013). In vitro studies showed that trehalose is able to stabilize enzymes, proteins, and lipid membranes, as well as protect biological structures from damage during abiotic stress. Trehalose has also been shown to protect cells and organelles from denaturation under in vivo conditions, but only when present at high concentrations (>10% of plant dry weight). For many years trehalose was thought to be limited to resurrection plants, as trehalose levels were only detectable in these plants (Li et al., 2011). Trehalose levels are nearly undetectable in higher plants to enable it to function as an osmoprotectant (Delorge et al., 2014; Redillas et al., 2012).

Transgenic plants have been genetically engineered to express *TPS* and/or *TPP* genes from microorganisms and they all exhibit some kind of increase in drought, salinity, or cold tolerance (Redillas et al., 2012). To demonstrate the physiological function of *TPS* the gene was introduced in rice. Overexpression improved the tolerance of rice seedlings to cold, high salinity, and drought without producing any other phenotypic changes. These results demonstrate that this *TPS* may improve the abiotic stress tolerance of plants by increasing the trehalose content and regulating the expression of environmental stress-related genes. Furthermore, the overexpression of *TPS*s from both classes increased plant tolerance to some common abiotic stresses (Li et al., 2011). For example, the substantial increase in proline, inositol and raffinose content in the leaves of *TPS1* transgenic potatoes appears to be a general response to drought stress (Kondrák et al., 2012). A reverse transcription PCR showed the expression divergence of the *TPS* gene family in rice under normal growth conditions and in response to stress (Yang et al., 2012).

Constitutive overexpression of yeast and *E. coli* *TPS* and/or *TPP* genes in various higher plants led to enhanced tolerance against abiotic stresses; however, some of these plants exhibited growth aberrations (i.e., stunted growth and pleiotropic changes). This is likely due to the unfavorable effects of the intermediate molecule of trehalose biosynthesis (T6P) during plant development (Lyu et al., 2013). This hypothesis seemed likely as combined overexpression of *TPS* and *TPP* (either separately or as a hybrid gene) did not produce these phenotypes (Delorge et al., 2014). Therefore, in order to minimize the accumulation of the intermediate metabolite in plant cells, a gene encoding a bifunctional fusion of *E. coli* *TPS* and *TPP* genes (*TPSP*) can be synthesized (Lyu et al., 2013).

Results suggest that the elevated production of trehalose through overexpression of the *TPSP* gene enhances the soluble sugar content and improves tolerance to abiotic stress in rice (Redillas et al., 2012). Transgenic tomatoes expressing *TPSP* accumulated higher levels of trehalose in their leaves, exhibited enhanced drought and salt tolerance, and demonstrated elevated photosynthetic rates under salt stress conditions. All transgenic plants had normal growth patterns and appearances (Lyu et al., 2013).

Exogenous osmoprotectants did not alleviate growth inhibition during saline stress, but they did have a large effect during growth recovery after stress. Exogenous trehalose increased the transcription of several antioxidant enzyme genes in rice, thus helping the plant to respond to abiotic stress (Nounjan and Theerakulpisut, 2012). T6P can also regulate other molecules. T6P interacts with sucrose nonfermenting related kinase-1 (SnRK1) and inhibits its activity. SnRK1 is most likely involved in the regulation of metabolism and growth in response to stress and by inhibiting its expression, T6P can promote crucial biosynthetic reactions. This regulation has consequences for crop production improvement (e.g., in the developing wheat grain and during the growth of potato tubers) (O'Hara et al., 2013). Martínez-Barajas et al. (2011) also observed this correlation in wheat and showed that the results were consistent with T6P tissue-specific regulation of SnRK1.

The trehalose pathway, and more specifically T6P, is also a key component of plant development in seeds and transition to flowering. When T6P levels are enhanced the expression of genes encoding enzymes for growth and biosynthetic pathways are also improved. The production of starch and cell walls can also be stimulated by T6P. Therefore, it is important that T6P levels be in equilibrium: not too high and not too low (Martínez-Barajas et al., 2011). It has also been established that the various TPS proteins can act together; for example, Zang et al. (2011) suggested that various *TPS* gene products can form complexes, modify T6P levels, and regulating plant development in rice.

5.2 Pyrroline-5-Carboxylate Synthases (P5CS) and Reductases (P5CR)

In food plants, proline is accumulated in response to environmental stresses (i.e., salinity, drought, heavy metals, and extreme temperatures). Proline is considered as an osmoprotectant as it stabilizes proteins and other cellular molecules, protects against denaturation, and scavenges ROS (Lv et al., 2011; Moustakas et al., 2011). There are two proline synthesis pathways in plants: the arginine/ornithine pathway, and the glutamate pathway (this is the predominant pathway in which proline is produced and accumulated in stressed cells). Glutamate is initially phosphorylated to γ -glutamyl phosphate and then reduced to glutamate- γ semialdehyde. This molecule is then cyclized to Δ^1 -pyrroline-5-carboxylate (P5C). These two steps are catalyzed by the bifunctional enzyme, P5C synthetase (P5CS). P5C is then reduced by P5C reductase (P5CR) to proline (Ben Rejeb et al., 2014; Giberti et al., 2014; Kim and Nam, 2013; Lv et al., 2011).

More studies have been performed with P5CS than P5CR. Karthikeyan et al. (2011) produced transgenic rice plants overexpressing *P5CS* from *Vigna aconitifolia* and noted salt tolerance after growing the plants under salt stress for 4 weeks. Chakraborty et al. (2012) observed that proline content increased with increasing levels of salinity in several genotypes of *Brassica* spp. This study suggests that salinity induced expression of *P5CS* (and its activity and proline accumulation) may serve as one mechanism of salinity tolerance in *Brassica* genotypes. Transcriptomic and proteomic analyses of potatoes under salt and cold stresses demonstrated increased gene and protein expression of several genes including *P5CSs* and *P5CRs* (Evers et al., 2012).

A *P5CS* gene from *V. aconitifolia* was genetically engineered in pigeon pea, and was found to improve proline accumulation under saline stress. The primary transgenic plants showed more proline accumulation than their nontransformed counterparts and levels of proline were elevated in transgenic plants when grown in the presence of 200 mM NaCl. Their growth performance was also enhanced and they showed lower levels of lipid peroxidation. This suggests that overproduction of proline may play an important role in salt tolerance (Surekha et al., 2014). The same *Vigna* gene was transferred to chickpeas via *A. tumefaciens*-mediated transformation. Heterologous expression of the *P5CS* gene improved the foods resistance to salt stress without reducing the plant yield (Kumar Ghanti et al., 2011).

A number of *P5CSs* can be found in food plants, although there are generally two (encoded by two duplicated plants). A novel *P5CS* was discovered in *Medicago truncatula* and this was found to be expressed at higher levels than the other isoforms under salt stress (Kim and Nam, 2013). *P5CS1* and *P5CS2* were cloned from sorghum and expression analysis revealed that both transcripts were upregulated after 10 days old seedlings were subjected to drought and saline stresses; expression levels of both *SbP5CS* genes were significantly enhanced after 3 days of drought stress. Therefore, the proline concentration improved after abiotic stress was correlated with *P5CS* expression. These results strongly suggest *P5CS1* and *P5CS2* are abiotic-stress inducible genes and both could be used to improve stress tolerance (Su et al., 2011).

In experiments with papaya, Zhu et al. (2012) showed that low (7°C) and high (35°C) temperatures induced *P5CS2* expression during storage and the increased expression of *P5CS2* preceded proline accumulation. In addition, the high temperature caused a more significant induction of *P5CS2* expression and a higher level of accumulated proline than the low temperature condition.

It is possible that there is differential regulation of different *P5CSs* in plants. Su et al. (2011) observed that the upregulated expression of *P5CS1* was higher than that of *SbP5CS2* in sorghum under drought and salt stress. Different stages of plant life also influence the gene expression of *P5CS* in foods; for example, the activity of *P5CS* was higher in the flowering stage than any other stage during higher salinity stress in *Brassica* (Chakraborty et al., 2012).

P5CS can be also being expressed in different plant organs; for example, [Su et al. \(2011\)](#) found that *P5CS2* was ubiquitously expressed in sorghum while *P5CS1* was mainly expressed in mature vegetative and reproductive organs. A study with Jerusalem artichoke (a moderately salt tolerant species) characterized proline regulation mechanisms under saline stress using two types of P5CS. Jerusalem artichokes were found to accumulate proline in roots, stems, and leaves during this stress. P5CS enzyme activities were increased whereas transcript levels of *P5CS2* increased in response to NaCl stress ([Huang et al., 2013](#)).

It is known that exogenous osmoprotectants cannot alleviate growth inhibition during abiotic stress; however, exogenous proline can help rice seedlings to recover from saline stresses and influence the expression of antioxidant enzymes ([Nounjan and Theerakulpisut, 2012](#)). Levels of expression are not equal between species or even between varieties of a same species; for example, researchers evaluated proline accumulation and the expression of *P5CS* in two varieties of soybeans under two concentrations of saline stress. The authors observed that gene expression levels varied between the two types, and thus concluded that the accumulation of proline is under the control of different mechanisms in the presence of salinity ([Çelik and Atak, 2012](#)). Similarly, the synthesis of proline was higher in two species of *Brassica* (out of four) under salt stress. Comparison of nucleotide and amino acid sequences showed conserved domains and inter- and intrageneric relatedness of these genes ([Chakraborty et al., 2012](#)).

Proline content may also be directly observed under environmental stress. The effect of cold acclimation and ABA treatment on the free amino acid composition of wheat was investigated. The amino acid content gradually increased during the 3 week cold acclimation period, while the effect of ABA became visible after only 7 days. ABA did not induce a major increase in the proline content, but it was greatly induced by cold. Corresponding alterations at the gene expression level were also found for proline. Since cold acclimation induced the accumulation of most amino acids (including proline), while ABA had a significant effect only on one amino acid, the cold-induced changes in proline levels were probably not mediated by ABA ([Kovács et al., 2011](#)).

Finally, as stated earlier, proline can also induce changes in antioxidant enzymes that act in response to abiotic stresses, and endogenous proline accumulation has a remarkable role in this regulation. [de Carvalho et al. \(2013\)](#) studied the expression four-antioxidant enzymes genes in *Swingle citrumelo* (ascorbate peroxidase, APX; catalase, CAT; superoxide dismutase, SOD; and glutathione reductase). All of the enzymes were differentially regulated in the leaves of transgenic citrumelos with high endogenous proline accumulation under normal water supply conditions and drought. Proline influenced the transcriptional activation of *APX*, *CAT*, and *SOD* during normal conditions, while mRNA levels of *APX* and *SOD* were influenced during drought. The research showed that, in addition to its known effects on various plants' physiological

and biochemical processes, high proline levels can also act as a regulatory/signaling molecule that is capable of altering the expression levels of important genes in relation to environmental stress.

6 Conclusions

Humankind depends on food crops as their main source of nutrients and energy for survival; however, most crops are cultivated in areas affected by environmental stresses. This situation is aggravated by climate change, which is gradually compromising global food security. Understanding the mechanisms of how plants respond to abiotic stress is a prerequisite for the development of genetically engineered food crops, which are focused on acquiring or increasing tolerance against abiotic stresses (i.e., drought, salinity, and high temperature). This chapter presented results from scientific studies that aimed to elucidate the genetic components (i.e., signaling molecules, transcription factors, and stress-induced genes) of the abiotic-stress response and their relationship with endogenous defense mechanisms of plants. Several molecules have been used to produce genetically engineered food crops with abiotic-stress tolerance, mainly via overexpression of genes of interest. However, additional studies are necessary to further identify new components of the plant stress response. It will also be important to consider how individuals in developing countries are going to get access to genetically engineered food crops.

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Detection of Prevented DNA Damage by Therapeutic Foods

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1 Introduction

As a long-term storage system for genetic data, the DNA double helix contains interlinked pathways that copy, modify, and maintain the entirety of the genome. Since the discovery of the DNA structure, and of the enzymes acting on it, researchers have developed significant biotechnology tools, such as cloning, subcloning, DNA sequencing, and amplification using polymerase chain reaction (PCR). However, more advanced research tools are needed to further understand the structural DNA changes that occur as a result of exogenous and endogenous factors.

Exposure to endogenous free radicals and exogenous mutagens can affect the stability of the genome and exposure to these carcinogens can result in degenerative diseases, such as Alzheimer's disease, cardiovascular disease, and cancer. Carcinogenic processes and the risk of cancer are related to high rates of DNA damage and chromosomal defects (Bonassi et al., 2000). When DNA is exposed to agents, such as ultraviolet (UV) irradiation and genotoxic chemicals, DNA damage occurs. In addition to these external factors, spontaneous DNA damage could happen at any time if normal cellular DNA processes fail and may result in oxidation, alkylation, cross-linking, dimerization, and DNA strand breaks (SBs). The normal metabolism of oxygen produces reactive oxygen species (ROS); however, excess amounts of ROS can cause oxidative damage in key cellular elements including lipids, DNA, and proteins. Research has shown that the majority of human degenerative diseases and the progression of aging are highly related to the imbalanced redox status induced by ROS.

Several micronutrients act as substrates that arrange DNA synthesis and repair in metabolic pathways (Chatterjee, 2001; Friso and Choi, 2002; Hageman and Stierum, 2001). When these nutrients are absent, a change in DNA structure and a deterioration in genomic integrity may be observed as DNA methylation causes a change in gene expression patterns.

The side effect profiles of many pharmaceuticals has led consumers to use food supplements for their healthcare needs. This has brought the definition of nutraceuticals (therapeutic foods)

into the world. A nutraceutical can be defined as a food that provides health and medical usefulness. Nutrients play an important role against environmental stresses and toxins and are able to prevent or lessen the impact of diseases. The reason that diets rich in fruits and vegetables are recommended is because they contain a significant amount of phytochemicals, which have antioxidant properties. Antioxidants help to overcome cell damage by reacting with free radicals and with these features they are able to reduce the risk of developing tumor cells becoming abnormal and reduce cell destruction. Of the dietary nutrients, polyphenolic compounds, such as flavonoids, carotenoids, terpenoids, and vitamin E and C protect the cells against damage. These nutrients are able to eliminate oxygen-derived free radicals, chelate redox-active metals, and inhibit lipooxygenase, thus they are able to prevent genotoxicity and improve genomic stability (Chatterjee, 2001; Dimitrios, 2006; Fenech, 2001). The following sections discuss and provide examples of the detection strategies of prevention properties of therapeutic foods on DNA damage.

2 DNA Damage and Aging

The causes of DNA damage in cells are varied. Environmental factors, such as radiation and UV light, as well as normal metabolic activities can cause DNA damage (Fig. 11.1). Every day, 1 million individual molecular lesions emerge as a result of DNA damage, many of which alter the transcriptional capabilities of the cells. This leads to damage of the DNA structure and produces mutations that are harmful to the cells (Fig. 11.2).

The huge majority of DNA damage (i.e., chemically modified bases) affects the primary structure of the double helix. There are two main types of DNA damage: endogenous and exogenous. Endogenous damage includes ROS attack. ROS are produced by normal cellular metabolism or external sources that are formed in cells. They react with DNA and generate genotoxic lesions, such as 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy), 8-oxo-7, 8-dihydroguanine (8-oxoG), 5-hydroxycytosine, and thymine glycol (TG). FaPy and TG are cytotoxic, while 8-oxoG residues are mutagenic. External agents, such as UV

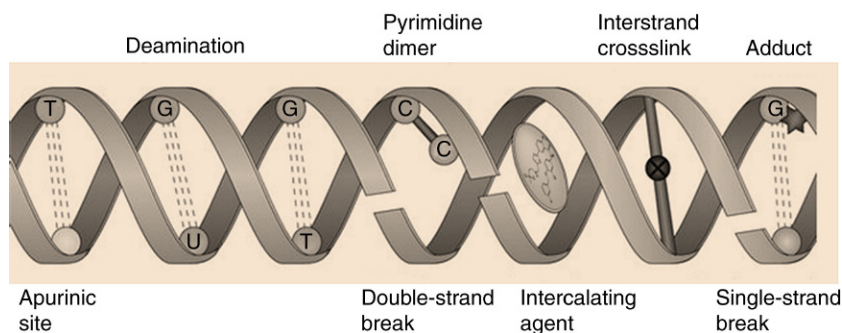


Figure 11.1: Types of DNA Damage.

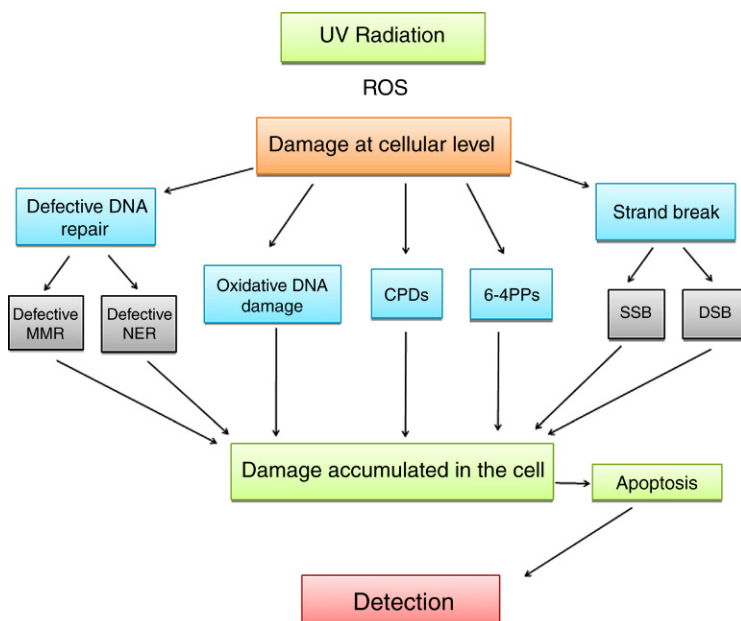


Figure 11.2: Schematic Representation of DNA Damage.

6-4PPs, Pyrimidine (6-4) pyrimidone photoproducts; CPD, *cis*, syn-cyclobutane pyrimidine dimer; DSB, double-strand breaks; MMR, mismatch repair; NER, nucleotide excision repair; SSB, single-strand breaks.

radiation, gamma and X-ray radiation, drugs, DNA-intercalating agents, and viruses also cause DNA damage (Figs. 11.1 and 11.2). Before the cell divides the damaged DNA may replicate leading to incorrect base pairing at damaged residues.

Scientific evidence showed that a large proportion of human degenerative diseases and the progression of aging are highly associated with an imbalanced redox status induced by ROS. Although a moderate amount of ROS is essential for maintaining cellular biological activities, excess ROS results in oxidative damage of key functional elements including cellular lipids, DNA, and proteins. Oxidatively stressed cells display several signs before the advancement of DNA damage, such as elevated ROS levels, mitochondrial perturbation, and accumulation of lipid peroxidation products.

Some common examples of ROS are superoxide anion (O_2^-), peroxy (ROO^-), peroxy radical ($ROO^\cdot$), ozone (O_3), hydrogen peroxide (H_2O_2), and reactive hydroxyl ($OH^\cdot$). Free radicals can easily react with other molecules because they are highly unstable. This triggers a chain reaction leading to free radical generation. The lipid-rich cellular membranes are injured by the damaging free radicals and lipid peroxidation can lead to membrane damage through the production of lipid-derived radicals. The excessive production of free radicals also leads to the depletion of antioxidants in vivo. This depletion can lead to oxidative stress, which may

Table 11.1: Potential DNA damage and the source of damage.

Sources of Damage	Potential Damages
Exposure to ionizing radiation	Abasic sites, oxidized bases, and fragmentation
Exposure to heat	Abasic sites, deaminated cytosines, oxidized bases, and fragmentation
Phenol/chloroform extraction	Oxidized bases
Exposure to light (UV)	Thymin dimers
Mechanical shearing	Fragmentation
Desiccation	Oxidized bases and fragmentation
Aqueous solution (storage)	Abasic sites, deaminated cytosines, oxidized bases, fragmentation
Formalin solution	DNA–DNA and DNA–protein cross-links

UV, Ultraviolet.

cause in cardiovascular diseases, liver diseases, cancer, diabetes, neurodegenerative diseases, and aging (Hochstein and Atallah, 1988).

2.1 Types of DNA Damage

There are different types of DNA damage, some of which are present in all extracted DNA, and some of which are only observed as a result of exposure to a specific source (see Table 11.1). The most common products of DNA damage are SBs, apurinic sites, 8-oxoguanine (a major biomarker of oxidative stress), and TG. Base deamination products may also be considered as a product of DNA damage.

In hydrolytic DNA damage, individual bases are totally removed or deamination occurs. Particular forms of DNA bases may be lost as a result of a mutagenic process producing apurinic/aprimidinic (AP) sites (Fig. 11.1), which can inhibit transcription if left unrepaired. Hydrolytic damage may be caused by the biochemical reaction of various metabolites or the overabundance of ROS. Oxidation is another type of DNA damage that causes alterations at bases, such as the transformation of guanine to 8-oxo-guanine (Hofer et al., 2006; Lindahl, 1993). This 8-oxo-guanine is a mutagenic product that can base pair with adenine and is considered to be as a significant factor in aging.

Other types of damages may emerge but only under certain conditions; for example, DNA–protein and DNA–DNA cross-links block the genetic investigation of stored samples. Although the cross-linking induced by formalin preserves structural morphology, cross-linked bases can hinder polymerases and DNA–DNA cross-links can curb denaturation. Moreover, the pH of formalin increases the rate of AP site formation and fragmentation.

Other types of DNA damage reported in the literature have occurred following exposure to UV light and H₂O₂ (Sinha and Häder, 2002). It is well-known that one of the most important causes of skin cancer is sunlight. When cellular systems are exposed to UVB (290–320 nm), pyrimidine dimers can form in the DNA, which can produce deletions, frame shifting, and

mutations. Characteristic lesions in UVB irradiated DNA include cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts. Mutations within the p53 tumor suppressor gene (Franklin et al., 1982; Miller, 1985) are characteristic of many basal cell carcinomas (Brash et al., 1991; Nakazawa et al., 1994; Pierceall et al., 1991).

Oliveira et al. (2007) used the alkylating agent, methyl methanesulfonate, to induce DNA damage. Damage caused by this substance emerged through the transfer of methyl groups to nitrogenous DNA bases, which led to alterations in base pairing. Chromium compounds are also carcinogens. Under in vitro conditions, Cr (III) is more reactive than Cr (VI) against DNA. Qi et al. (2000) examined the ability of Cr (III) to induce oxidative DNA damage in calf thymus DNA incubated with CrCl₃ and H₂O₂ through the formation of 8-hydroxydeoxyguanosine (8-OH-dG).

2.2 Detection of DNA Damage

Detection of DNA damage is important in the pharmaceutical industry, genotoxicity testing, toxicology, and in the diagnosis of genetic disorders. Some strategies that are commonly used to detect DNA damage include PCR, comet assay, terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) assay, high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), HPLC-electrospray tandem mass spectrometry (HPLC-ESI-MS), HPLC-electrochemical detection (HPLC-EC), fluorescence in situ hybridization (FISH), flow cytometry (FCM), immunological assays (e.g., immunofluorescent and chemiluminescence thymine dimer detection), immunohistochemical assays, enzyme-linked immunosorbent assays (ELISAs), and electrochemical methods, (Kumari et al., 2008).

PCR is a reliable and commonly used technique for detecting DNA damage. Although PCR-based assays are sensitive and effective at measuring gene-specific DNA damage, they fail to quantify and recognize the type of damage.

Single cell gel electrophoresis (or the comet assay) is a commonly-used, sensitive, and low-cost technique for evaluating DNA damage or repair. It helps to measure the single- and double-strand breaks (DSBs), the base or base-pair damages, AP sites, DNA cross-links, and apoptotic nuclei in the cells. This detection system can be applied to cultured cells, solid tumors, peripheral blood, cancer cells, yeast cells, sperm, and bacteria. The main stages of the comet assay consecutively involve the lysis of cells, electrophoresis, and fluorescent staining of DNA. Damaged DNA is visualized as a fluorescent stain or comet formation (Fig. 11.3).

The tail moment and tail length are two important quantitative parameters for evaluating the DNA damage. In cases where an increase in damage has occurred, the tail moment will increase. Computerized image analysis is used to detect this rise.

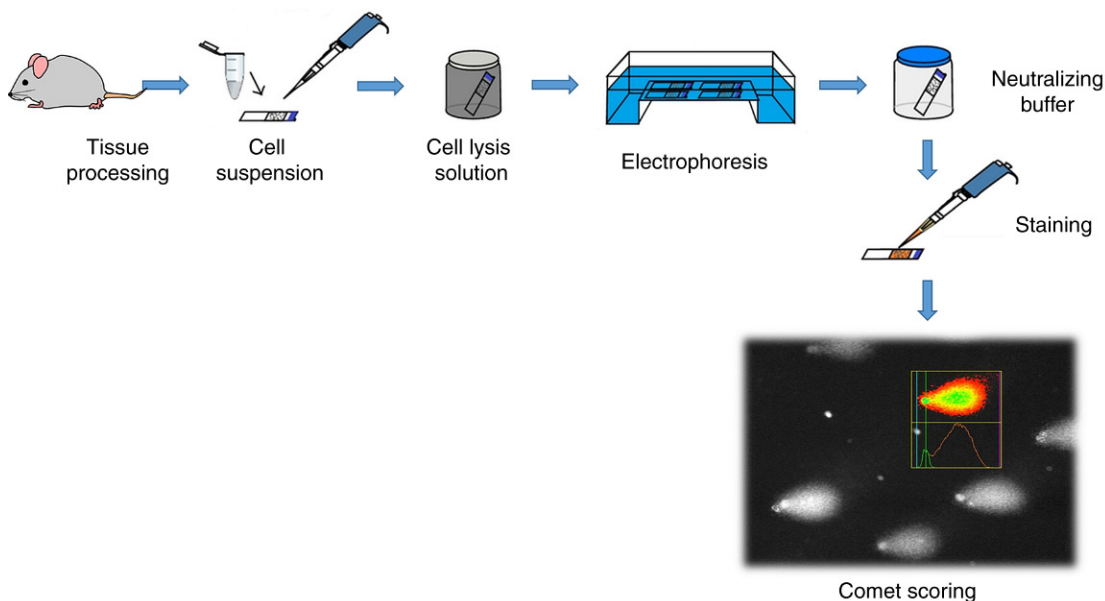


Figure 11.3: Schematic Representation of the Comet Assay Protocol.

There are two comet assay formats: (1) alkaline comet is used to analyze SSBs and is performed under denaturing conditions, and (2) neutral comet is used to analyze DSBs and is carried out under nondenaturing conditions.

When providing the results of the single gel electrophoresis experiments, the percentage of total cell DNA is given. For a normalized measure of the percentage of total cell DNA, the integrated tail intensity $\times 100$ is divided by the total integrated cell intensity. A damage measure combining the amount of DNA in the tail with the distance of migration, $\Sigma (L_x \cdot \% \text{DNA}_x)$.

HPLC-ESI-MS is a sensitive and specific assay (Frelon et al., 2000). The ionization property of electrospray is a convenient and preferred method for evaluating DNA adducts (Rindgen et al., 1995). The detection of *cis*-syn and *trans*-syn I cyclobutane thymine dimers and UV-induced dimeric pyrimidine photoproducts can also be realized using this method (Douki et al., 2000). A urinary 8-hydroxylated species of guanine has been determined by HPLC-ESI-MS. These species are ribonucleoside, 8-hydroxylated base, deoxynucleoside, and the corresponding nonoxidized species.

Nonisotopic marking with FISH is used to detect the relative location and copy number of disturbed DNA content in nuclei or chromosomes (Murthy and Demetrick, 2006). FISH is more sensitive at detecting abnormal urinary cells than FCM, and requires very little material (Sauter et al., 1995). Changes of HER-2/neu (c-erbB-2) in patients with breast cancer have been detected using this assay (Sidoni et al., 2006).

FCM has been developed as a method to detect apoptosis (Koopman et al., 1994). This assay is also used for the detection of DNA strand breakage, chromosomal aberrations, chemical–DNA adducts, and sister chromatid exchange (Bickham et al., 1994; Lower and Kendall, 1990). Nucleotide excision repair has also been detected using an alkaline unwinding FCM assay (Thyagarajan et al., 2007). In this method the detection was made by measuring the DNA content of nuclei labelled with propidium iodide.

In ELISAs antibodies and color changes are used to identify substances. A solid-phase enzyme immunoassay is used to detect the presence of a particular substance (i.e., an antigen) in a liquid sample.

The ability of GC–MS to detect DNA base products means that it is commonly-used to detect oxidative DNA damage whereby polar bases have been transformed into thermally-stable derivatives. However, the oxidative damage related to the derivatization of hydrolyzed DNA can sometimes be overestimated when using this method at higher temperature and in the presence of air. In these situations the quantity of 8-OH-guanine, 8-OH-adenine, and 5-OH-cytosine have been seen to be increased (Jenner et al., 1998).

Electrochemical methods provide sensitive detection and selective miniaturized devices for DNA damage (Paleček et al., 1998; Paleček and Jelen, 2002). DNA is electroactive; adenine, cytosine, and guanine are reduced at mercury electrodes while guanine and adenine are oxidized at carbon electrodes. Altered current in these electroactive bases could be one reason for DNA damage (Fojta, 2002, 2004; Paleček et al., 2002). In cells exposed to ionizing radiation, 8-oxoguanine could be detected by its oxidation signal at carbon electrodes (Brett et al., 2000); however, thymine dimers could not be detected (Fojta, 2002, 2004). Voltammetric detection can be used to complement DNA damage detection techniques, such as the comet assay. HPLC-EC detection is both selective and sensitive, which makes it an ideal tool to measure low levels of 8-oxoguanine in cells.

Chemiluminescence has been widely used to monitor free radicals and as a sensitive assay to detect reactive metabolites in organs, cells, and enzyme systems (Guo et al., 2007).

In the halo assay, fluorescent propidium iodide (PI) is used to intercalate into the DNA helix. This causes a change in the supercoiling status of the DNA. DNA can be visualized based on the changing diameter with increasing PI concentration. Conversely, the TUNEL assay detects DNA fragmentation through fluorescing free ends of the DNA molecule. Hence apoptosis can be detected via fluorescent microscopy (Bruggeman et al., 1997). Both detection strategies can also detect single SBs and DSBs, although the sensitivity and specificity of this method is limited.

2.3 Repair of DNA Damage

Over 200 human genes are known to be involved in DNA repair and many factors, such as the age of the cell, cell type, and environment influences also have a role in repair activity.

The major mammalian DNA repair pathways can be classified as following:

1. Base excision repair (BER)
2. DNA mismatch repair (MMR)
3. Nucleotide excision repair (NER)
4. DNA strand break repair pathways:
 - a. Single-strand break repair
 - b. Double-strand break repair

In a single SB (i.e., when only one strand of the double helix is defective), the other strand can be used as a guide for correcting the damaged strand. Three excision repair pathways can repair single-stranded DNA damage: NER, BER, and MMR. When endogenous and exogenous DNA damage occurs, BER and NER are used. The types of lesions repaired by BER include oxidative lesions (8-oxo-G), deoxyuracil, various alkylation products, and spontaneous depurination. Despite the extraordinary fidelity of DNA synthesis, errors do occur. Such errors can be detected and repaired by the postreplication MMR system. MMR can only aid replication fidelity if the repair is targeted to the newly synthesized strand. The types of lesions repaired by NER include UV pyrimidine photodimers, which recognizes the bulky lesion that block DNA replication.

DNA DSBs are the most lethal form of DNA damage. By multiple genomic changes, DSBs might result in significant DNA damage, such as amplifications or deletions. Moreover, they may result in enduring cellular damage, which can cause malignancies (Ciccia and Elledge, 2010; Helleday et al., 2007; Moynahan and Jasin, 2010).

Current standard cancer therapies and agents targeted to cancerous tissues may also be damaging to noncancerous tissues. Therefore there is an urgent need for new therapies that have minimal toxicity to healthy tissues. Vitamins C and E are known antioxidants and both DNA oxidation and chromosome damage may occur due to a lack of these vitamins (Claycombe and Meydani, 2001; Fraga et al., 1991; Halliwell, 2001). Oxidative modification of guanosine is known to be inhibited by vitamin C (Fraga et al., 1991), and vitamin E has been reported to scavenge lipid ROO radicals, thus inhibiting DNA single SBs caused by ROS (Tsuda et al., 2000). The protective effects of vitamin E have also been linked to its ability to inhibit the formation of hydroxyl radicals (HO) (Machlin, 1990).

Vitamin D is also known for its antioxidant properties. It has been shown to prevent the breakage of double-stranded DNA by curbing iron-dependent lipid peroxidation (Chatterjee, 2001; Wiseman, 1993).

It is well-established that ROS can lead to DSBs in cancer cells. In the present study, the results showed that selenocysteine can induce ROS, but not in normal human fibroblasts (Chen and Wong, 2009). Therefore cancers that are unable to repair DSBs may be sensitive to treatment with selenium compounds. Sirtuin 1 is a nicotinamide adenine dinucleotide-dependent

deacetylase. Resveratrol may activate these enzymes that are known to activate DNA repair (Poulsen et al., 2013).

Niacin may also play an important role in DNA repair. Niacin is the precursor of NAD⁺, the substrate of poly(ADP-ribose) polymerase (PARP). PARP is thought to be important for the regulation of many cellular processes involving DNA repair and programmed cell death. Enzymes such as this are able to reduce the risk of mutations that cause long-term cancer (Hageman and Stierum, 2001). Daily diets containing B3 (50 mg), B2 (10 mg), and coenzyme Q10 (100 mg) increased the activity of PARP and improved gene repair in women with breast cancer. Hence niacin content may be critical for preventing cell death and maintaining genome integrity.

Vitamin B9 (folate) and B12 deficiency may also damage chromosomes (Fenech, 2001). The genome-wide gene expression profile can be affected by changes in DNA methylation. Folate has a direct role in DNA methylation as it acts as a methyl donor in its main circulating form, 5-methyltetrahydrofolate (Friso and Choi, 2002). In times of folate deficiency, genomic DNA methylation is decreased.

3 Therapeutic Foods (Nutraceuticals)

Nutraceuticals are chemicals found as natural components in foods or other ingestible products (Table 11.2). They can help to protect the human body, be used to treat one or more types of disease, or enhance physiological performance.

Research in the field of functional foods and nutraceutical is growing. Some studies have focused on the use of nutraceuticals in the prevention or treatment of diseases, such as atherosclerosis and degenerative joint diseases (Table 11.3). What distinguishes nutraceuticals from functional foods is vague but the main difference between the two rests upon the form of consumption. For example, functional foods are consumed as ordinary foods, while nutraceutical are consumed as capsules, pills, or tablets. Thus, a phytochemical included in a food formulation is considered as a functional food, while the inclusion of the same phytochemical in a capsule makes it a nutraceutical (Rajat et al., 2012).

Nutraceuticals are used to provide functional benefits to the organism by increasing the supply of natural building blocks. These building blocks can be used to diminish signs of a disease or to improve performance. Nutraceutical on the market today consist of both traditional foods and nontraditional foods. Traditional nutraceuticals are natural whole foods, such as the lycopene (in tomatoes) and omega-3 fatty acids (in salmon). Conversely, nontraditional nutraceuticals are foods produced through agricultural breeding, which have added nutrients and ingredients to enrich their nutritional values (i.e., β -carotene-enriched rice and cereals with added vitamins or minerals).

Table 11.2: Classification of the sources of nutraceuticals.

Plants	Animals	Microbes
β-Glucan	Linoleic acid	<i>Saccharomyces boulardi</i>
Ascorbic acid	Eikosapentaeonik asit	<i>Bifidobacterium bifidum</i>
γ-Tocotrienol	Docosahexaenoic acid	<i>Bifidobacterium longum</i>
Luteolin	Sphingolipid	<i>Bifidobacterium infantis</i>
Cellulose	Choline	<i>Lactobacillus acidophilus</i> (LCI)
Lutein	Lecithin	<i>L. acidophilus</i> (NCFB 1748)
Gallic acid	Calcium	<i>Streptococcus salivarius</i> (subs. thermophilus)
Indole-3-carbinol	Coenzyme Q10	
Pectin	Selenium	
Glutathione	Zinc	
Potassium	Creatine	
δ- Limonene	Mineral	
Genistein		
Lycopene		
Hemicellulose		
Lignin		
Capsaicin		
Geraniol		
α-Tocopherol		
β-carotene		
Selenium		
Mineral		

Cui et al., 2004 established that plant antioxidants have a complementary effect when they are taken together. They also observed that plant extracts may contain a number of antioxidants with unknown complementary activities. It is important that the biological activities of known or potential nutraceuticals are detected as it helps in the understanding of how these nutraceuticals interact with each other and with ROS. The biological activity (i.e., the antioxidant activity) of an extract isolated from a natural source could be measured by quenching ROS or by increasing the levels of ROS production. And, the other detection system, offers a convenient and reliable approach for measuring prevented DNA damage by therapeutic foods based on conformational and structural DNA changes.

4 Detection of Prevented DNA Damage

Cancer is a complex disease that is variable in its presentation, development, and outcome. The same heterogeneity and variability exists at the cellular and molecular level. Carcinogenesis or tumorigenesis is the initiation of cancer formation, whereby normal cells are transformed into cancer cells through a progression of changes at the cellular, genetic, and epigenetic level. This ultimately leads to cells being reprogrammed to undergo uncontrolled cell division.

To block chemically induced carcinogenesis, the phase I enzymes, which are involved in detoxification reactions, are inhibited. Almost all procarcinogens are bioactivated by

Table 11.3: Classifications of nutraceutical (especially with antioxidant properties).

Classes of Components	Components	Potential Benefits	Sources
Carotenoids	Lycopene	Antioxidant activity; protective effect against prostate, bladder, cervical cancer, and leukemia	Tomatoes, pink grapefruit, guava papaya, and water-melon
	Lutin	Anticancer (colon) activity	Corn, avocado, egg yolk, and spinach
	β -Carotene	Antioxidant activity	Carrots and tomatoes
	α -Carotene	Antioxidants activity and anticarcinogenic	Carrots
	α - Cryptoxanthin	Antioxidant and anticancer activities	Oranges and tangerines
Polyphenolic compounds	Flavonones	Antioxidant and anticancer activities	Citrus fruits
	Flavones	Antioxidant and anticancer activities	Vegetables, fruits, and soybeans
	Anthocyanins	Antioxidant, anticancer, and antiinflammatory activities (inhibits the proliferation of prostate cancer)	Blueberries, blackberries, and black raspberries
	Phenolic acids	Reduced formation of cancer	Berries and legumes
	Resveratrol	Antioxidant activity and prevents breast cancer	Red wine, dark grapes, raisins, berries, and peanuts
	Curcumin	Antioxidant	Promising chemopreventive and anticancer agents
	Quercetin	Antioxidant, antiinflammatory, antiproliferative, and antiangiogenic activities	Many fruits, grains, and vegetables
Isothiocyanates	Sulforaphane	Enhances detoxification and encourages cellular antioxidant defenses	Cauliflower, broccoli, cabbage, kale, and horseradish
Dietary fibers	Soluble fiber	Anticancer activity and helpful in maintaining the digestive tract	Legumes, oats, and some fruits
	Insoluble fiber	Anticancer (colon) and helpful in maintaining the digestive tract	Whole grain foods Wheat, corn bran, and nuts
Phenols	Caffeic acid	Encourages cellular antioxidant defenses	Apples, pears, citrus fruits, and some vegetables
	Ferulic acid	Reduce the risk of coronary heart disease	Stanol ester dietary supplements
Tocotrienol	Isoprenoids	Anticancer (breast) and promotes cardiovascular health	Grains and palm oil
Phytoestrogens	Isoflavones (genistein and daidzein)	Prevents prostate and breast cancer and induces apoptosis	Soy beans and legumes

(Continued)

Table 11.3: Classifications of nutraceutical (especially with antioxidant properties). (cont.)

Classes of Components	Components	Potential Benefits	Sources
Noncarotenoid terpenoids	Lignans	Inhibits the development of breast and colon cancer	Flaxseed, rye, and vegetables
	Perillyl alcohol	Anticancer activity	Cherries and mint
	Saponins	Reduces cholesterol levels in blood	Legumes (chickpeas, fenugreek, and all pulse crops)
Alkaloids	Terpenol	Anticancer activity	Carrots
	Terpene limonoids	Anticarcinogenic	Peels and membranes of citrus fruits
	Tropane alkaloids	Treatment of heart disease	Solanaceous members including deadly night shade and datura
Minerals	Vincristine and vinblastine	Antineoplastic	periwinkle
	Calcium and potassium	Important constituents of a balanced diet	Food
	Selenium	Important constituent of a balanced diet	Food
	Zinc	Important constituent of a balanced diet	Food
Glucosinolates	Copper	Important constituent of a balanced diet	Food
		Anticancer (bladder) activity	Cruciferous vegetables and cauliflower
Saponins		Lowest cholesterol levels and has anticancer activity (colon)	Chickpeas and soybeans
Fatty acids	Omega-3 fatty acids	Controls inflammatory processes and supports the maintenance of brain function	Salmon and flaxseed
Anthraquinones	Monounsaturated fatty acids	Reduces the risk of coronary heart disease	Tree nuts
	Capsaicin	Anticancer, antiinflammatory, and antiapoptotic activities	Capsicum (hot peppers)
Vitamins	Vitamin E (α -tocopherol)	Prevent the alternation of nitrites and nitrates to nitrosamines and has antioxidant activity	Germinated seeds and fish oils
	Vitamin C (ascorbic acid)	Protects human cells from endogenous oxidative DNA damage	Fruits (oranges)

drug-metabolizing enzymes into reactive intermediates that can bind to DNA and protein. These events are primarily catalyzed by cytochrome P450 (CYP) enzymes. The second phase of the carcinogenesis process involves phase II detoxification enzyme systems. Phase II enzymes include glutathione-S-transferase (GST), epoxide hydrolase, ferritin, and NAD(P)H:quinone oxidoreductase 1 (NQO1).

A large number of dietary supplements also protect against carcinogenesis. Their anticarcinogenic properties have been attributed to their ability to bind toxicants and upregulate carcinogen-metabolizing enzymes. Various constituents of the human diet also act as antioxidants and help to prevent the increased formation of free radicals generated by the toxicants. Nutraceuticals have been used to describe nutrients that are able to significantly and positively affect human health. More than half of all current drugs originate from natural products and studies suggest that nutritional products may be active in cancer chemoprevention. Some natural compounds (i.e., curcumin, resveratrol, indole-3-carbinol, 3, 3-diindolylmethane, sulforaphane, and brassinin) are used in cancer treatments and in chemoprevention. In this section the detection strategies for prevented DNA damage by therapeutic foods will be explained.

4.1 Phenolic Compounds

Flavonoids are polyphenolic compounds that have a wide range of antimicrobial, antiinflammatory, antiallergenic, antioxidant, and anticancer activities. Flavonoid-rich foods (i.e., fruits, particularly grapes) modulate the activities of enzymes that participate in the activation (phase I) and detoxification (phase II) of procarcinogens. Cancers that are related with this hormone are inhibited by flavonoid-mediated modulation of sex steroid hormone receptors (Virgili and Marino, 2006; Whitehead and Rice, 2006). These results may explain the relationship between the dietary consumption of flavonoids and the incidence of hormone-related breast, prostate, testicular, and colorectal cancers (Amin and Buratovich, 2007; Galluzzo and Marino, 2006).

Quercetin is a natural bioflavonoid found in many fruits, vegetables, leaves, and grains. It possesses diverse pharmacologic effects and acts as an antioxidative, antiinflammatory, antiproliferative, and antiangiogenic agent. The PI3K/AKT/mTOR pathway is an intracellular signalling pathway that is important in cell cycle regulation. At nontoxic concentrations quercetin is able to inhibit AKT and mTOR.

Curcumin is derived from *Curcuma longa*. It is a yellow spice and phenolic compound that is used to prevent cancer (Bar-Sela et al., 2010). Indole-3-carbinol is found at high levels in cruciferous vegetables. This compound is a natural hydrolysis product of glucobrassicin and it has been shown to block tumor cell proliferation (Marconett et al., 2012).

Red wine is rich in resveratrol and this compound is thought to inhibit CYP 450 1B1 (Peter Guengerich et al., 2003). The inhibition of the P450 enzyme may mean that resveratrol could help to prevent breast cancer in humans (Damianaki et al., 2000).

The anticancer properties of green tea are related to the presence of epigallocatechin gallate (EGCG) and other polyphenols. EGCG suppresses androgen receptor expression and signalling. Flavonoid-rich black tea and green tea are produced from leaves of the same

plants, but the difference between them occurs postharvesting from the leaves of processing shape. Black tea is produced as a result of oxidation during the fermentation process. Zhao and Shah assessed the influence of tea samples, with or without fermentation, on several markers of oxidative damage (Zhao and Shah, 2016). The oxidative DNA damage effects were studied in a normal human epithelial colon cell line using the comet assay and the temperature during electrophoresis was kept constant at 4°C. The results of the DNA damage were given as the percentage of DNA in the tail, total DNA damage was counted as the sum of SBs and oxidized lesions, and cellular DNA repair capacity (RC) was calculated using the equation:

$$RC = 100 \times (DD_{t=0} - DD_{t=30}) / DD_{t=0}$$

where $DD_{t=0}$ represents DNA damage before the recovery period and $DD_{t=30}$ represents DNA damage after a 30 min recovery. Results showed that fermented tea samples were more effective than nonfermented samples and they were able to stimulate DNA repair, resulting in fewer SBs and oxidative lesions.

Fig. 11.4 illustrates that the protective effects of tea treatment were more evident in normal cells than in carcinomas. The RC in the normal colon cells achieved 42.5 and 30.1% when preincubated in fermented tea phenolic mixture (fTPM) and fermented black tea, respectively. Most significantly, lactic acid bacteria-fTPM reduced total DNA fragmentation by 37.2% before repair and by 52.9% after repair. DNA damage reached the maximum level (i.e., 99% DNA in the tail) in positive control cells, while the results were 42.4%–62.0% for cells pretreated with tea samples (Fig. 11.4). Black tea samples protected cells against H_2O_2 -induced DNA damage by directly inhibiting SBs and inducing DNA repair mechanisms. The results suggest that preincubation with black tea improved the endogenous repair system in addition to directly preventing DNA damage.

Anthocyanin-rich natural products belong to the flavonoid family of photochemicals and have been shown to protect against DNA damage (Hou et al., 2004). Singletary et al. demonstrated that an anthocyanin-rich extract from concord grapes could inhibit benzo[a]pyrene (B[a]P)-induced DNA adduct formation in immortalized human breast epithelial cells. This inhibition was associated with enhanced activity of phase II detoxification enzymes [GST and NAD(P)H:quinone reductase] and altered activity of ethoxyresorufin-O-deethylase (EROD). EROD activity is a catalytic measurement of CYP1A induction. Concord grape extract at 5 and 10 $\mu\text{g/mL}$ increased EROD activity by 25 and 45%, respectively, compared to controls. Treatment of cells with concord grape extract at 10 and 20 $\mu\text{g/mL}$ significantly enhanced cytosolic GST activity by 21 and 25%, respectively, compared to controls (Singletary et al., 2007).

Plant-derived antioxidants [i.e., green tea polyphenols (GTPP)] are effectively used to protect against diseases that result from oxidative damage. Jiang et al. (2001) assessed the protective

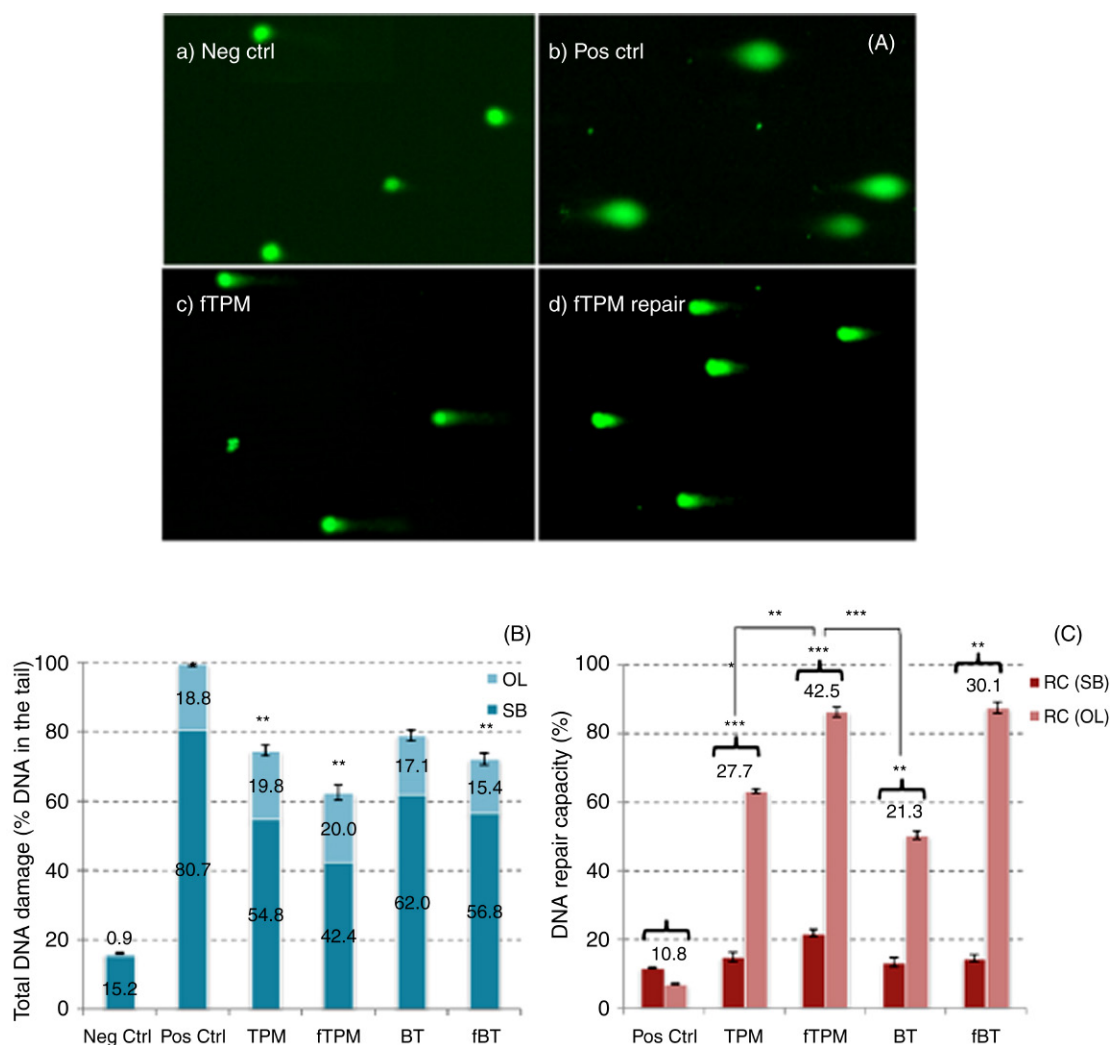


Figure 11.4: Effect of Black Tea Sample Pretreatment on H₂O₂-Induced DNA Damage and Repair in CCD 841 Cells.

(A) Representative DNA comets from cells (a) without treatment, (b) treated with H₂O₂ directly, (c) pretreated with fTPM before exposure to H₂O₂, and (d) treated with fTPM and H₂O₂ and repaired for 30 min; all samples were digested with Fpg after cell lysis. (B) DNA SB and OL, Fpg-sensitive site. (C) DNA RC; overall RC of each treatment group is indicated below the brace; significant at (**) $P < 0.01$ and (***) $P < 0.001$ compared with the Pos Ctrl; asterisk on top of braces indicate significant difference from fTPM-treated samples. BT, Black tea extract; fBT, fermented black tea extract; fTPM, fermented TPM; fMRS, fermented MRS broth; OL, oxidative lesions; Pos Ctrl, positive control; RC, repair capacity; TPM, tea phenolic mixture. Reprinted from Zhao, D., Shah, N.P., 2016.

Synergistic application of black tea extracts and lactic acid bacteria in protecting human colonocytes against oxidative damage. J. Agric. Food Chem. 64, 2238–2246, with permission.

effects of GTPP on tributyltin (TBT)-induced oxidative damage. TBT has been extensively used in boat paints due to its antifouling properties and the harmful effects of TBT on marine organisms have been determined earlier. Between the TBT treated group and the control group there was a statistically significant difference in both tail length and tail moment. Single gel electrophoresis data revealed that the number of cells with DNA damage was significantly higher in untreated mice compared to GTPP-treated mice. The results showed that GTPP was effective at reducing DNA damage both *in vivo* and *in vitro*. GTPPs ability to scavenge ROS and prevent DNA breaks means that the protective mechanism may be monitored. GTPP could be an effective food supplement for reducing the cytotoxicity of TBT (Liu et al., 2008).

Pomegranates are rich in anthocyanidins and polyphenols and they have traditionally been used in folk medicine. Ellagitannins, which are commonly found in berries and pomegranates, are bioactive polyphenols with anticancer, antioxidative, and antiinflammatory activities. Ellagitannin-rich pomegranate extract inhibited the proliferation of prostate cancer cells and blocked tumor-associated angiogenesis (Sartippour et al., 2008). Studies showed that the pomegranate juice was able to reduce the levels of prostate specific antigen (PSA) after primary treatment with surgery or radiation. Guo et al. analyzed the ROS scavenging ability and protective effect of pomegranate extracts on DNA damage using the CuSO_4 -Phen-Vc- H_2O_2 -DNA chemiluminescent method *in vitro*. Time-course analysis of the chemiluminescent emission showed an initial small peak within 3 min and a second peak 3.5 min after the addition of H_2O_2 . The emission intensity slowly reduced as the extract concentration was increasing. The results showed that pomegranate extract had considerable antioxidant activity and prevented DNA damage ($P < 0.05$). When comparing the IC_{50} values (half maximal inhibitory concentration), the authors found that white pomegranate seed extract was the most effective at preventing DNA damage (IC_{50} 3.67 $\mu\text{g/mL}$) (Guo et al., 2007).

Many studies have demonstrated that honey contains flavonoids, phenolic acids, (Alvarez-Suarez et al., 2010, 2012), ascorbic acid (AA), proteins, and carotenoid. Zhou et al. studied the protective effects of buckwheat honey on HO-induced DNA damage, determining the antioxidant activity (i.e., HO-scavenging activity, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), reducing power assays and chelating), the total phenolic content, and the individual phenolic acid contents. The phenolic acids (gallic, protocatechuic, caffeic, *p*-coumaric, and *p*-hydroxybenzoic acids) were analyzed by HPLC while the effect of honey on the HO-induced DNA damage was evaluated by gel electrophoresis. The results showed that honey samples prevented HO-induced DNA breaks by 21%–78% compared with control value (Zhou et al., 2012). This study proves the antioxidant effect of buckwheat honey.

Mate (MT) is a popular beverage in South America and it has traditionally been used as a medicine. Yerba MT is rich in phenolic acids, caffeine, and saponins, all of which act as free radical scavengers. Miranda et al. (2008) studied the antioxidant activity of MT and the ability of this substance to influence DNA repair in male mice. After having been randomly

assigned into four groups, the animals were given MT tea extract (0, 0.5, 1.0, or 2.0 g/kg) for 60 days. Following the intervention, kidney, liver, and bladder cells were isolated and DNA damage was analyzed using the comet assay. The DNA repair process was also investigated by the same methodology. The authors showed that the ordinate alimentation of MT tea increased the resistance of DNA to H_2O_2 -induced DNA SBs and improved the DNA repair.

In another presented study, MT was studied to have antioxidant properties in vitro and in vivo (Leonard et al., 2010). More specifically, these authors studied the effect of an MT infusion on hydroxyl and superoxide radicals. Electron spin resonance experiments indicated that MT scavenged HO $\cdot$ s and superoxide radicals at all tested concentrations ($P < 0.05$). Malondialdehyde and comet assay data ($P < 0.05$) also showed that MT could inhibit HO-induced lipid peroxidation and DNA damage in a dose-dependent manner in a leukemia virus-transformed cell line. Comet assay results were obtained after exposing the cells to the Fenton reaction for 1 h (10 μ M $FeSO_4$ + 100 μ M H_2O_2). Treatment with MT (15.5, 31, and 310 μ g/mL) significantly reduced tail length in a concentration dependent manner. Therefore, these results showed that MT had significant antioxidant effects against hydroxyl and superoxide radicals both in chemical and cell culture systems.

Another study assessed the effect of virgin olive oil phenolic extract (OOPE) on mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage in immortalized HeLa cells (Erol et al., 2012). DNA damage was evaluated in mitochondria and two nuclear regions using quantitative PCR assay. Cells were pretreated with OOPE for 4 h before DNA damage was determined. The results showed that OOPE totally inhibited nDNA damage under stress conditions while mtDNA damage was not affected by this procedure. The results propose that OOPE has a protective effect against nDNA damage.

Genistein is an isoflavone that can curb the proliferation of breast cancer cells and prevent colon cancer (Barnes et al., 1995; Pagliaci et al., 1994). Genistein inhibits the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Li et al., 2005), promotes apoptosis, and alters polyamine metabolism (Linsalata et al., 2005). Wei et al. (1996) studied the effect of the soybean isoflavone genistein on 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation in calf thymus. The authors found that genistein curbed the formation of 8-OHdG induced by UV light or the Fenton reaction in a dose-dependent manner. The levels of 8-OHdG and deoxyguanosine (dG) were measured using HPLC-EC in a reverse phase C18 (5 μ m, 250 $\times$ 4.6 mm) column. The pH of the eluent buffer was 5.1, and the buffer contained 25 mM sodium acetate, 15% methanol, 12.5 mM citric acid, 30 mM NaOH, and 10 mM acetic acid. The levels of 8-OHdG and dG were calculated from the peak area based on their corresponding standards. At a genistein concentration of 5 μ M, almost 95% of UV light-induced 8-OHdG was inhibited while Fenton reaction-induced 8-OHdG was quenched by almost 20%. The potent inhibition of UV light-induced oxidative DNA damage suggests that genistein has a potential anticarcinogenic role in photocarcinogenesis.

Singh et al. studied the antimutagenic activity of red onion peel on pUC18 plasmid DNA damage (pUC18 is a plasmid cloning vector commonly used with *Escherchia Coli*) induced by the Fenton reagent. Five extracts of red onion peel were analyzed for their total phenolic content, antioxidant activity, free radical-scavenging activity, flavonoids, antiradical power (assayed by DPPH radical), and reducing power (expressed as AA equivalents/mL). The EA fraction showed antimutagenic activity by dose dependently inhibiting HO-induced nicking in plasmid pUC18 DNA. The plasmid DNA was exposed to Fenton's 40 reagent in the absence and presence of the extracts and the linear form of the plasmid was monitored using horizontal gel electrophoresis. The results showed that oxidative stress was reduced in the presence of different concentration (2–10 µg/mL) of EA (Singh et al., 2009a). The presence of quercetin, kaempferol, and ferulic, gallic, and protocatechuic acids were analyzed by HPLC and MS–MS. The results suggested that EA extracts of red onion peel could be used as a natural antioxidant nutraceuticals.

Green tea is one of the most widely consumed hot beverages and it has significant chemopreventive qualities. Macedo et al. (2012) assessed DNA damage in the presence of green tea extract using the comet assay on individual cells. The authors found that cells treated with green tea extract demonstrated significantly reduced tail moments when compared to positive control cells, thus demonstrating that green tea extract protects against DNA damage and has the potential to be used as a nutraceutical.

Svobodova et al. (2009) assessed the ability of the phenolic fraction of *Lonicera caerulea* (LC) and *Vaccinum myrtillus* (VM) fruits to reduce UVB-induced damage in HaCaT cells (an immortal keratinocyte cell line from adult human skin). The authors found that fruit extracts were able to actively reduce the extent of DNA breakage (especially at 25 and 10 mg/L). LCE and VME significantly decreased ROS generation (reactive oxygen and nitrogen species) and LCE pretreatment prevented the proliferation of keratinocytes. Pretreatment of keratinocytes with phenolic fruits prevented DNA single SB formation and pre- and posttreatment with phenolic fruits significantly reduced single SB at 25 and 10 mg/L of LCE and VME, respectively.

4.2 β -Glucan

β -Glucan-rich functional foods can prevent the development of cancer. Foods such as barley, mushrooms, cereals (mainly oats), algae, and yeasts contain significant amounts of β -glucan, which is predominantly found in their cell walls. In vivo studies have shown that the low-molecular weight β -glucan is effective at preventing mutagenic effects when animals were exposed to cyclophosphamide. Oliveira et al. (2007) examined the mechanism of action of β -glucan in Chinese hamster ovary (CHO), wildtype (k1), and DNA repair-deficient (xrs5) cells using β -glucan from *Saccharomyces cerevisiae*. Four different doses of β -glucan were tested for their antigenotoxic, antimutagenic, and cell viability effects in CHO-k1 and CHO-xrs5

cells. In the CHO-k1 cell line, β -glucan had a chemopreventive effect in all the protocols tested: pretreatment showed a 35.0%–57.3% reduction, simultaneous treatment showed a reduction of 19.7%–55.6%, simultaneous treatment with preincubation showed a reduction of 42.7%–56.4%, and posttreatment produced a 17.9%–37.6% reduction. The comet assay showed that β -glucan does not have a genotoxic or antigenotoxic effect. The results of the cell viability tests demonstrated that β -glucan preserves cell viability in both cell lines while preventing apoptotic events. These results suggest that β -glucan could be used in drug development and β -glucan in foods could provide nutraceutical characteristics.

Angeli et al. (2009) examined the chemoprotective effect of β -glucan extracted from the mushroom *Agaricus blazei* against DNA damage using the comet assay. The mechanism of β -glucan action was tested in three treatment protocols: simultaneous, pretreatment, and presimultaneous. The greatest effect was observed in the presimultaneous treatment, which could be explained by the inhibitory effect of β -glucan on enzymes of the P450 family. The results showed that β -glucan does not exert genotoxic or mutagenic effect, but that it does protect against B[a]P-induced DNA damage in all protocols. The data suggest that β -glucan acts by binding B[a]P or by capturing free radicals produced during its activation (Angeli et al., 2009).

4.3 Vitamin E

α -Tocopherol, a form of vitamin E, is an antioxidant, which means that it can help the body fight off a range of degenerative conditions. α -Tocopherol absorbs in the UVB region, which may account in part for the ability of topically applied α -tocopherol to suppress UVB-induced damage. McVean and Liebler reported that the topical application of naturally occurring α -tocopherol was effective at preventing UVB-induced DNA damage (McVean and Liebler, 1997). UVB irradiation in mice induces the production of thymine dimers in the epidermal DNA. Dimer formation is a relevant parameter for evaluating the efficiency of chemopreventive molecules and can be performed using capillary gas chromatography with electron capture detection. In these experiments the temperature was held isothermally at 100°C for 1 min and then programmed at 12°C/min up to 280°C where it was held for 32 min. The dimer eluted at ~25 min and the limit of quantitation was 1 fmol of the injected dimer. The authors found that the topical application of α -tocopherol inhibited dimer formation and a significant inhibition was observed when α -tocopherol concentrations were 1% or more (w/w). The maximum reduction observed was 84% less than controls, which was obtained at 10% (w/w) α -tocopherol. UVB irradiated mice were also treated using commercial sunscreens with increasing sun protection factor (SPF) ratings and significant protection was seen with SPF 15 and 30 ($P < 0.05$ and $P < 0.01$, respectively).

Park et al. (2014) studied the protective effect of orange (*Citrus × aurantium*) on DNA damage. Antioxidant potential was examined by a series of measurements such as

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, oxygen radical absorbance capacity, total radical-trapping antioxidant potential, and cellular antioxidant activity.

Determination of the protective effects of orange flesh (OF) and orange peel (OP) extract on H_2O_2 -induced DNA damage were performed using the comet assay. Human leukocytes were preincubated with 0, 1, 10, or 50 $\mu\text{g/mL}$ of OF or OP extracts for 30 min. The authors found that DNA damage in cells pretreated with 50 $\mu\text{g/mL}$ of OP or OF extract was significantly lower than that of cells treated with H_2O_2 . For each of the extracts the maximum protective effect of pretreatment was obtained with the 50 $\mu\text{g/mL}$ dose. The high antioxidant activity of OP suggests that this could be used as a nutraceutical.

4.4 Fermented Papaya

Fermented papaya preparation (FPP) has been shown to be a natural antioxidant that is able to prevent lipid peroxidation *in vitro* and *in vivo*. [Marotta et al. \(2006\)](#) examined the effect of this novel nutraceutical on leukocyte DNA in elderly patients. The study group consisted of healthy elderly patients who were given either 9 g of FPP/day or a placebo for 3 months. DNA was isolated and purified from leukocytes before being analyzed using a HPLC-EC detection system. Levels of 8-OHdG were also detected in these samples using UV absorbance. The results were expressed as the ratio of the absorbance peak of 8-oxodGuo adducts to that of dGuo adducts $\times 10^5$. The FPP-supplemented group showed a significant increase in antioxidant protection with glutathione S-transferase M1 (GSTM1) (–) and of plasma DNA adduct, irrespective of the GSTM1 genotype. The results showed that there was a 60% decrease in leukocyte 8-OHdG concentration when GSTM1 (–) participants were treated with FPP. Therefore FPP is a promising nutraceutical for enhancing antioxidant defense in the elderly.

[Rimbach et al. \(2000\)](#) examined the protective effect of FPP on supercoiled plasmid DNA against Ferric nitrilotriacetate (Fe-NTA) and H_2O_2 -induced single SBs and DSBs. DNA damage was determined using the comet assay and similar protective effects of FPP were evident when human T-lymphocytes were challenged with Fe-NTA or H_2O_2 . FPP dose-dependently inhibited Fe-NTA and H_2O_2 -induced DNA damage. EPR spin trapping studies demonstrated that antioxidant properties of FPP emerged as a result of its hydroxyl-scavenging and iron-chelating properties.

4.5 Vitamin C

[Fraga et al. \(1991\)](#) established that dietary AA reduced the levels of 8-oxoG in human seminal fluid and protected human sperm from endogenous oxidative DNA damage. [Giri et al. \(1998\)](#) also examined the protective effects of AA against cisplatin-induced sperm head abnormalities and chromosomal aberrations. The oxidized nucleoside 8-OHdG was measured in human sperm DNA using HPLC-EC. The potentials for electrodes 1 and 2 were adjusted to +0.10 and +0.30 V, respectively. This study was performed in two groups: a group of

20–50 years old free-living individuals and a group provided with an AA content controlled diet. In the first group high levels of 8-OHdG were correlated with low plasma concentrations of AA. When dietary AA was decreased from 250 to 5 mg/day, the level of 8-OHdG in sperm DNA increased by 91%. In the second group, an increase in AA from 5 to 250 or 60 mg/day for 28 days reduced the 8-OHdG by 36%. These results indicate that dietary AA protects human sperm against endogenous oxidative DNA damage.

4.6 Carotenoid

Carotenoids are a group of tetraterpenoids. Of the naturally occurring carotenoids, lycopene is the most efficient ROS quencher (Miller et al., 1996). It has significant antioxidant activity and is predominantly found in vegetables and red fruits. Studies have shown that the consumption of lycopene may have a preventive affect against human prostate cancer (Giovannucci, 2007; Mills et al., 1989) and lycopene can block cell growth in breast, prostate, and endometrial cancer cells by inhibiting NF- κ B activity (Hung et al., 2008). Additional studies (Gann et al., 1999; Giovannucci et al., 1995) with the other antioxidant nutrients have demonstrated that a lower risk of prostate cancer is only related with increased lycopene intake. The study showed that consumption of almost five portions of tomato products/week was related to a 40% lower risk of prostate cancer in males in the United States (Gann et al., 1999). Chen et al., examined the effects of a tomato sauce-based dietary intervention on oxidative DNA damage and PSA levels in patients with prostate adenocarcinoma (Chen et al., 2001). The tomato sauce amounting to 30 mg of lycopene/day was consumed for 3 months and serum and prostate lycopene concentrations, serum PSA levels, and leukocyte DNA oxidative damage were assessed before and after the nutritional intervention. HPLC-EC with a Coulochem II detector was used for the analysis. The peak potential of 8-OHdG was detected at +350 mV and the mobile phase was adjusted to pH 5.2 and included 100 mM sodium acetate and 5% methanol. The authors found that dietary lycopene intake significantly increased the serum and prostate lycopene concentrations from 638 to 1258 nM ($P < 0.001$) and 0.28 to 0.82 nmol/g ($P < 0.001$), respectively. The quantity of oxidative DNA damage was calculated as 8-OHdG/ 10^5 dG. Following lycopene intake the leukocyte DNA damage was found to be significantly reduced (0.61 8-OHdG/ 10^5 dG–0.48 8-OHdG/ 10^5 dG). Furthermore, prostate tissue oxidative DNA damage was also significantly lower in men who received the intervention (0.76 8-OHdG/ 10^5 dG) compared with randomly selected patients (1.06 8-OHdG/ 10^5 dG; $P = 0.03$). Serum PSA levels also decreased from 10.9 to 8.7 ng/mL after dietary lycopene intake. These data confirm a possible role for lycopene in the treatment of prostate cancer.

Pink guava is a tropical fruit that is rich in lycopene. In the present study, solvent extract at 25 μ g/mL (2.32 μ M lycopene) and SC-CO₂ extract at 200 μ g/ml (5.09 μ M lycopene) had protective effect against H₂O₂-induced cytotoxicity (Kong et al., 2010). However, when the authors used the alkaline comet assay they established that only high concentrations of

solvent extract (lycopene = 18.65 μM) or lycopene standard (10 μM) were able to protect cells against DNA damage. Comet tails were observed following H_2O_2 treatment thus demonstrating the presence of cellular DNA. Reduced comet tails were observed for the negative control, the solvent extract, and the lycopene standard. The results showed that the protective effect of decanted pink guava against cell damage was due to the presence of lycopene.

4.7 Multicomponent

Allium sativum (garlic) and *Allium cepa* (onion) are commonly used flavoring agents. Numerous studies have shown that organosulfur compounds demonstrate antioxidative, antimutagenic, antiproliferative, and apoptotic effects. Garlic and onion products are rich in organosulfur compounds, with diallyl disulfide and diallyl sulfide (DAS) from garlic and dipropyl disulfide (DPDS) and dipropyl sulfide from onion appearing to be the most active molecules of *Allium* species. The aim of this study, by the Comet assay, was to evaluate the effects of isothiocyanates, organosulfurs, and vitamin C against human hepatoma cells (Arranz et al., 2007). The authors found that the organosulfur compounds reduced DNA SBs and that DAS and DPDS (5 μM) had protective effects against DNA SBs (37 and 35%, respectively). In addition, OSCs decreased the levels of oxidized pyrimidines but not purines. Isothiocyanate compounds (i.e., phenethyl isothiocyanate, allyl isothiocyanate, and indole-3-carbinol) and vitamin C showed protective effects toward DNA SBs and oxidized purine and pyrimidine bases; 0.1 μM phenethyl isothiocyanate reduced the oxidized purines by 41% while 1 μM of allyl isothiocyanate and indole-3-carbinol showed a protective effect against oxidized pyrimidines, although allyl isothiocyanate was more effective with a 56% reduction. The highest reduction (51%) of oxidative DNA damage was obtained using 1 μM of indole-3-carbinol. These findings suggest that OSCs, ITCs, and vitamin C may be protective against DNA SBs and oxidative DNA damage due to their free radical-scavenging efficiency.

Vitamin E, melatonin, and ascorbate are free radical scavengers that inhibit the formation of 8-OH-dG in dose-dependent manner. By Qi et al., the ability of melatonin to diminish Cr (III)-induced oxidative DNA damage in vitro was studied (Qi et al., 2000). Examining the formation of 8-OH-dG in calf thymus DNA incubated with CrCl_3 and H_2O_2 , HPLC-EC was used to detect 8-OH-dG and the authors found that the oxidative DNA damage was reduced by 50% when using 0.51, 30.4, and 36.2 μM of melatonin, ascorbate, and vitamin E respectively. The results also show that melatonin is almost 70-fold more effective than ascorbate or vitamin E and are consistent with melatonin's highly protective effects against Cr (III).

By Singh et al., the aqueous fruit extract (FE), leaf extract (LE), and seed extract (SE) of *Moringa oleifera* to inhibit oxidative DNA damage and antioxidant potentials (Singh et al., 2009b). The results showed that these extracts could significantly inhibit the $\text{OH}^\cdot$ -dependent damage of pUC18 plasmid DNA. HPLC-mass spectroscopy was used to analyze

chlorogenic acid, ferulic acid, vanillin, kaempferol, gallic acid, quercetin, and ellagic acid. LEs were found to have better antioxidant activity (85.77%) and higher total phenolic content than FEs, SEs, or the α -tocopherol standard. LEs also showed better results than the other extracts in terms of protein oxidation, OH-induced deoxyribose degradation, lipid peroxidation inhibition, and superoxide anion-scavenging capabilities.

Strawberries are important dietary components as they contain high contents of vitamin C, folate, and phenolic constituents (especially anthocyanins). These bioactive compounds are natural antioxidants and contribute to the high nutritional quality of the fruit. The present study, the methanolic extracts from the *Sveva* cultivar for their anthocyanin content and their ability to protect human dermal fibroblasts against UVA radiation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and the comet assays (Giampieri et al., 2012). HPLC-ESI-MS identified five anthocyanin pigments. Furthermore, the extract of strawberry demonstrated a photoprotective activity in fibroblasts following UVA radiation. In comparison to control cells, strawberry-treated fibroblasts showed diminishing DNA damage and increased cellular viability following high-UV treatments. To clarify the DNA protective role of strawberry extracts, the comet assay was used on control cells and cells preincubated with strawberry. Cells pretreated with 0.25 and 0.5 mg/mL of strawberry extract showed a highly significant decrease ($P < 0.05$) in DNA damage compared with the controls. These results suggest that strawberry has photoprotective activity and may protect skin against the adverse effects of UVA radiation.

Slamenová et al., 2011 studied the effects of rosemary oil (RO) consumption against the DNA-damaging effects of oxidative agents in rat testicular cells (TCs). The activity of the antioxidant enzymes glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were measured spectrophotometrically while DNA lesions were detected using the comet assay. FCM was used to calculate the DNA-damaging effects of H_2O_2 and DMNQ in haploid, diploid, and tetraploid TCs. The results showed that oxidative DNA lesions were significantly reduced in TCs from rats nourished with RO; however, the activity of antioxidant enzymes did not differ between TCs from control and RO-supplemented rats. When DNA damage was induced by an increase in H_2O_2 in control cells, an increase in DNA damage was detected. However, the levels of DNA damage were significantly lower in rats nourished with progressively increased RO concentrations (Fig. 11.5). All doses of RO significantly reduced the levels of both DNA breaks and oxidative DNA damage.

5 Conclusions

Current standard cancer therapies and agents targeted to cancerous tissues may also damage noncancerous tissues. Therefore novel treatments are required that do not harm normal cells.

Due to the side effect profiles of pharmaceuticals, many consumers have begun to use alternative food supplements as healthcare products. In addition, a large number of dietary

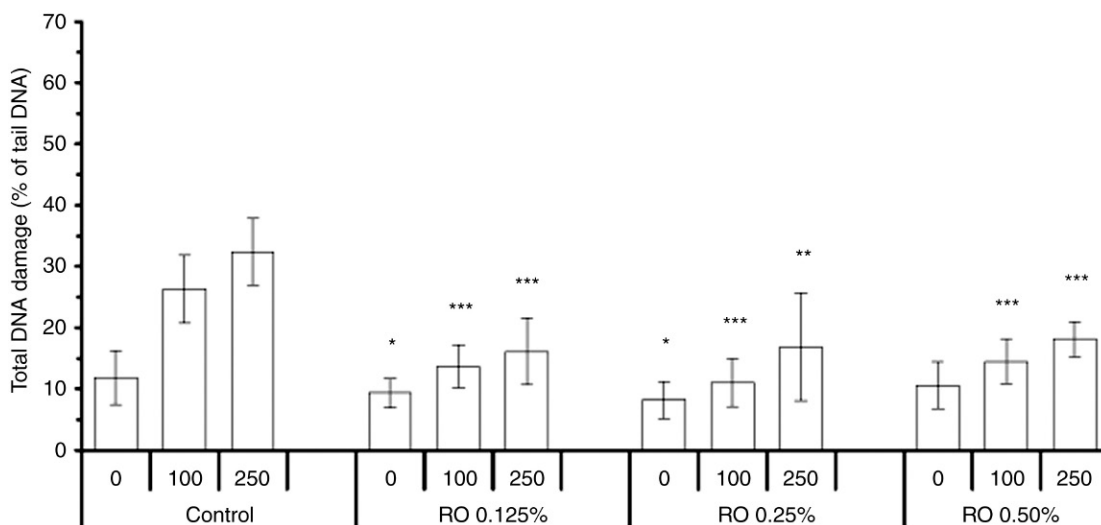


Figure 11.5: Level of DNA Damage in TCs Isolated From Rats Supplemented Either With Cremophor EL (control) or with 0.125, 0.25, and 0.50% Rosemary Oil (RO).

Freshly isolated TCs were treated with different concentration of H₂O₂ (0, 100, and 250 μM). Data represent means ± SD of 3–5 isolations. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 indicate significant differences compared to the control. Reprinted from Slamenová, D., Horváthová, E., Kováčiková, Z., Kozics, K., Hunáková, L., 2011. Essential rosemary oil protects testicular cells against DNA-damaging effects of H₂O₂ and DMNQ. *Food Chem.* 129, 64–70, with permission.

supplements can provide protection against carcinogenesis. Various constituents of the human diet can also act as antioxidants and prevent the increased formation of free radicals generated by the toxicants. Scientific evidence suggests that the vast proportion of human degenerative diseases and the progression of aging are highly associated with an imbalanced redox status induced by ROS. Although a moderate amount of ROS is essential for maintaining cellular biological activities, excess ROS results in oxidative damage in key cellular elements including lipids, DNA, and proteins. The innate antioxidant defense systems, consisting of enzymatic and nonenzymatic components, plays an essential part in combating ROS-induced oxidative stress in cells.

The term nutraceutical has been established to describe nutrients that are able to significantly and positively affect human health. A number of laboratories have reported that some dietary nutrients have protective effects against cancer. Among these dietary micronutrients, flavonoids and carotenoids could play an important role in the prevention of cancer development.

In this chapter, the mechanism through which therapeutic foods are able to prevent DNA damage have been discussed. Studies suggest that the most commonly used techniques to detect DNA damage are single gel electrophoresis, HPLC-EC, and HPLC-ESI-MS. The most common

products of DNA damage are SBs, apurinic sites, 8-oxoguanine, TG, and base deamination products. HPLC was used to detect and measure 8-OHdG using comparison control groups while single gel electrophoresis and comet assays were used to measure the percentage of damaged DNA. In these studies the results were given as compared control groups.

Studies have demonstrated that carotenoids and flavonoids have significant protective effects against the DNA damage induced by some chemicals or UV radiation. These findings indicate the potential of dietary nutrients for human therapies and the applicability of genotoxic evaluation for improving health. Such experiments could contribute to a higher quality nutritional life in the future.

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Genetically Engineered Crops: Opportunities, Constraints, and Food Security at a Glance of Human Health, Environmental Impact, and Food Quality

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1 Introduction

Poverty and hunger are the main troubles in developing countries and generating sufficient food is likely to become a major problem in the future. Many factors contribute to poverty and hunger, such as improper infrastructure, the ruling government, political instability, and environmental and economic conditions, therefore one cannot say that science or genetically modified (GM) crops will entirely fulfill food needs. Keeping this in mind, we do not claim that GM crops alone will “feed the world.” Humankind are being faced with the challenge of feeding billions of people and in the near future we will need to be able to produce vast quantities of food; however, even if sufficient food is generated, many people will still go to bed hungry. Hence the foremost question should be why does hunger exist? In answer to this question, reasons could include inadequate dealings in agriculture, climate change, unseasonable weather, and food wastage; however, it should be noted that pathogen and insect attack and drought and irrigation problems are important biotic stresses that are responsible for approximately 50% of yield losses (up to 90% in some cases) in crop plants, such as cotton, sunflower, pulses, ornamentals, and cereals (Kissoudis et al., 2014). Abiotic factors also contribute to yield losses and insect pests remain a major reason for crop loss despite the extensive use of chemical insecticides (i.e., organophosphates, synthetic pyrethroids, organochlorines, carbamates, and biopesticides). The continued use of pesticides may lead to environmental, agricultural, medical, and socioeconomic issues. Although farmers use various synthetic pesticides to manage field pests, insects are developing resistance to these

toxins. To develop effective toxin-resistance management strategies in the future, a complete understanding of the physiological and genetic mechanisms by which insects become resistant to these insecticidal proteins or phytochemicals is needed. Information about adaptation mechanisms and metabolic pathway regulation in polyphagous insects will pave the way for the development of new pest-control strategies. To this end, transgenic crops expressing *Bacillus thuringiensis* (*Bt*) Cry proteins are the most significant and commonly used strategy against insect pests (Li et al., 2004); however, insect resistance to Cry1Ac and Cry1Ab has led some to question the significance of transgenic crops.

Food production yield can be increased through the use of GM crops. Plants are sessile and rely on their defense against biotic or abiotic stresses to survive under natural conditions. One of the oldest methods to overcome the adverse effects of stress is to transform the plant through the insertion of a foreign gene of plant or animal origin. The protein encoded by the gene will confer a particular trait or characteristic to the plant. Stresses, such as salinity, drought, pathogens, and pest attacks can be overcome using this technology. A decline in the quality of agricultural land, increasing temperatures, and elevated CO₂ emissions have all occurred due to human activity (Peters et al., 2011). This has led to an increase in the incidence of drought and ultimately an increase in soil salinity (Munns and Tester, 2008; Zhao and Running, 2010). This ongoing situation is leading toward climate change and may be supporting the spread of insect pests and pathogens. An increase in the number of virulent strains also increases the chances of crop damage (Garrett et al., 2006). Connections between climate change and biotic and abiotic stresses are being observed more frequently and the current situation indicates that worse situations may be yet to come.

Recombinant DNA technology has brought revolutionary changes to the field of medicine and introduced novel methods for treating diseases and delivering drugs. Insulin is a hormone that is responsible for controlling blood glucose levels in humans. If an individual cannot produce enough insulin they will suffer from diabetes; however, by using recombinant DNA technology scientists have been able to produce human insulin (using bacteria as a host cell) and insulin therapy is now commercially available. Vaccines are developed by injecting a suspension of weak (or dead) pathogenic cells into the body to enhance the production of antibodies against the antigen of interest. Viral vaccines against herpes, influenza, hepatitis, and foot and mouth disease have all been developed using this technology. The production of human growth hormone using recombinant DNA technology has been used to treat dwarfism. Monoclonal antibodies are produced using hybridoma technology, which produces unlimited antibodies in culture. These antibodies are used to produce vaccines against different viral infections. Recombinant DNA technology can be used to produce interferon in *Escherichia coli*, which can be used to treat cancer-like hairy cell leukemia. Antibiotics are commonly used to treat all types of bacterial infections and diseases and they can be produced by microorganisms, as well as in the laboratory. Alexander Fleming discovered penicillin in 1928 using recombinant DNA technology.

Recombinant medicines and industrial products (i.e., monoclonal antibodies, vaccines, plastics, and biofuels) are now also being developed by GM technology. For these reasons, GM technology has attracted considerable media attention.

Environmental activists (the anti-GMO lobby) have accused proponents of this technology of pushing GMOs into agriculture without adequately considering the health and environmental risks. Public opinion must be considered before this new technology is used or released in the field; however, both sides have taken to blaming the public for their lack of understanding. Anti-GMO campaigners demand that there should be zero risk from GM crops while pro-GMO campaigners ask, is there any proof that GM crops might be harmful when consumed? Lack of awareness appears to be the key problem and hence there is a need to educate the public, but after imparting information about GM crops, both the pro- and anti-GMO lobbyists must leave the general public to accept or reject this technology. This chapter focuses on agricultural GM crops and their environmental impacts: positive and negative. Transgenic plants (*Bt*) have been grown around the globe for the last 20 years and there have been no harmful effects of GM crops on the ecology; however, GM plants have still not been universally accepted in agricultural systems. This may be due to the general public being afraid of GM crops and fearing a repeat of the time when the overuse of pesticides poisoned the environment and created the storm by “Silent Spring.” As such, environmental extremists, a few farmers, and the general public believe that GM plants contain genes that can wreck the environment and may be harmful to humans.

2 Green Revolution

The green revolution (GR) can be described as an amalgamation of research, development, and technology-transfer steps that transpired between the mid-1930s and the late 1960s to increase agricultural production in the developing world (Hazell, 2009). The GR arose from the adoption of new technologies, such as the use of dwarf wheat and high-yielding varieties (HYVs) of rice alongside the use of chemical fertilizers, agrochemicals, and a controlled irrigated water supply. Mechanized cultivation has also played a crucial role in the GR (Farmer, 1986). Norman Borlaug is known as the father of the GR, and he saved billions of people from starvation by developing HYVs of cereal grains. Furthermore, he also expanded the irrigation infrastructure, modernized management techniques, and distributed hybridized seeds, fertilizers, and pesticides to farmers. He was honored with the Nobel Peace Prize in 1970. The Mexican government, the United Nations, and the Rockefeller Foundation were the first to transform agricultural productivity using irrigated cultivation. In 1943 the Mexican government founded the International Maize and Wheat Improvement Center (CIMMYT), which acts as a base for international agricultural research (Barkin, 1997). The International Rice Research Institute (IRRI) in the Philippines has also performed pioneering work in the development of HYVs of rice. In India, alongside the efforts of the Indian minister of agriculture (C. Subramaniam) and Norman Borlaug, the Ford Foundation has helped to

import wheat seeds from the CIMMYT. Punjab was selected as the region for these trials and alongside HYVs of seeds, other agronomical practices helped to initiate the GR in India.

GM strategies to increase yields includes the selection of genotypes with higher yield potential, wide adaptability, short growth duration, resistance to biotic stresses, tolerance to abiotic stresses, and superior grain quality. These elite genotypes were conventionally bred to improve yield-associated traits and plants were selected on the basis of their reduced height, increased tiller number, and erect (not droopy) leaves. The yield potential of maize was increased using these selection criteria (Johnson et al., 1986). Varietal improvements have been achieved by increasing the harvest index by changing the plant phenotype from tall and leafy with weak stems to short stature and high tillering with dark green and erect leaves. This enables the plant to use solar energy more efficiently and possess sturdy stems (Rajaram and van Ginkel, 1996). Moreover, adaptation to a wider climatic zone better suits the global acceptance and the photoperiod plays a major role in adaptation. The photoperiod insensitivity gene (*se1*) was introduced to the IR8 variety of rice, which was released in 1966 by the IRRI. This transgenic rice produced double the yield of previous rice varieties when grown in irrigated conditions as it was more responsive to fertilizers. Another strategy to increase the yield involves developing varieties that only take a short time to grow. The incorporation of early flowering (*ef*) genes in rice reduced the maturation time from 130 to 105 days (Khush, 1987). As less crop varieties are vigorously commercialized and cultivated, there is a reduction in the genetic diversity of these crops (Khush, 1977) and this has led to an increase in disease incidence and insect numbers. Therefore efforts have been concentrated on developing germplasms that could act as possible sources for disease and insect resistance (Khush, 1989). Germplasms were screened for resistance and parents (donors) with resistance to the main diseases were identified (Khush, 1992). The first multiresistant rice to be developed by the IRRI was IR26. Another strategy to increase yields is to develop cultivars that can cope with abiotic stresses, such as drought, flooding, salinity, nutritional deficiency, or toxins. A dominant gene, submergence tolerance 1 (*Sub1*), and several quantitative trait loci that improve submergence tolerance, have been identified in the FR13A variety of Indian rice (Mackill et al., 1993).

Although the GR contributed to widespread poverty reduction, forestalled hunger for millions of people, and circumvented the conversion of thousands of hectares of land into agricultural cultivation, a large proportion of undeveloped and developing countries saw that poverty and food insecurity persisted. Studies have since shown that agricultural productivity has a profound effect on the rate of poverty reduction (Hazell, 2010), and as the GR strategy was based on intensification of favorable areas, its contribution to poverty reduction in the marginal production environments was relatively low. Despite the increase in productivity, micronutrient intake is still lagging behind (Webb, 2009). HYVs that were more responsive to external inputs were central to achieving productivity; however, in many cases the appropriate research and policies to incentivize judicious use of inputs were largely lacking

(Pingali, 2007). The limitations of the GR can be overcome by GM crops by theoretically transferring any candidate gene from any source without genepool limitations.

3 GM Crops: An Overview of Human Health, Their Environmental Impact, and Food Quality

3.1 Food Quality and Nutritional Content

The demand for agricultural products is growing on a daily basis but natural resources and ecosystem conditions are stagnant; however, meeting the increasing demand for food is not the only problem. It is also important to address the quality of food, more specifically the nutritional content. Hence attention has increasingly turned toward policy makers, researchers, and development organizations. Scientists have planned and calculated how to construct a plant with enough vitamins, minerals, and proteins to provide poor and malnourished individuals with a day's worth of nutrition in a single meal. The nutritional content of food is of global concern, both in terms of total energy intake and vitamin content to prevent complications related to micronutrient deficiencies. Abnormality, depravity, and mortality worldwide are due to dietary micronutrient deficiencies, such as a lack of vitamin A, iodine, iron, or zinc. Vitamin A deficiency is responsible for 500,000 cases of irreversible blindness and up to 2 million deaths each year (Christou and Twyman, 2004), with pregnant women and children being most prone to this deficiency (WHO, 2001).

GM technology offers a way to engineer plants to express additional products that can combat malnutrition (Fig. 12.1). Low dietary intake of iron, vitamin A, iodine, and zinc creates micronutrient deficiencies that lead to many health complications in humans, mainly in children and pregnant women. Vitamin A deficiency is prevalent among the poor whose diets are based on rice or other carbohydrate-rich, micronutrient-poor calorie sources. Rice does not contain any vitamins or provitamins and as it is the predominant food source in the world, rice-based diets can lead to vitamin deficiencies. These deficiencies not only affect function but they ultimately progress to cause compromised immune systems, which greatly increases the risk of common childhood infections. Southeast Asia and Africa are prone to these types of complications. Golden Rice has the potential to complement existing efforts that seek to reduce blindness and other induced diseases. To reduce the levels of vitamin A deficiency, efforts have been taken to produce vitamin A capsules; however, small countries, such as Nepal and Ghana require USD 2 million/year to run these campaigns. The unaffordable price has meant that countries, such as India have been unable to run these programs across the whole country. It is known that dietary supplementation of vitamin A can eliminate vitamin A deficiency and the "Golden Rice Project" provided a solution by generating rice containing large amounts of vitamin A. It has been estimated that 72 g of dry Golden Rice 2 will provide 50% of the RDA of vitamin A for a 1–3 year old child (Paine et al., 2005). This project therefore offers a long-term sustainable solution.

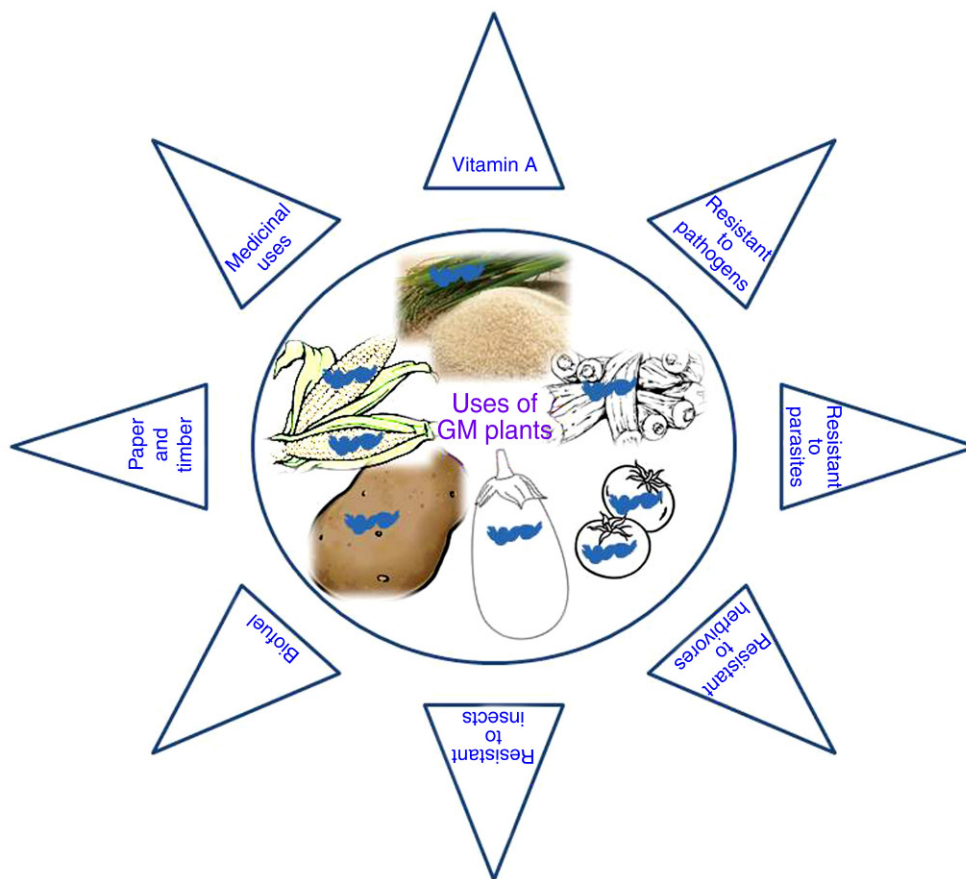


Figure 12.1: Benefits of GM Plants.

GM technology offers a way to engineer plants to produce more micronutrients that can combat malnutrition. GM plants can increase nutrient levels, produce pharmaceutical molecules and have improved resistance to disease, cold or drought, thus increasing food security. Abnormality, depravity and mortality worldwide are due to dietary micronutrient deficiencies, such as the lack of vitamin A, iodine, iron, or zinc can be overcome by GM crops. Besides medicinal, paper and timber use and to combat biotic and abiotic stresses can be done by modified plants.

Vitamin E lowers harmful blood cholesterol levels, inhibits the growth of breast cancer cells, helps to maintain cardiovascular health, and has antioxidant properties. Vitamin E deficiency can cause neuromuscular problems, anemia due to oxidative damage of red blood cells, retinopathy, and impairment of the immune response. [Cahoon et al. \(2003\)](#) at the Donald Danforth Plant Science Center in Missouri produced a common type of field corn that contained sixfold more vitamin E than normal. The BioCassava Plus Project run by an international multicenter research team led from Ohio State University, produced GM cassava with sufficient vitamins, minerals, and proteins. The success of this study in greenhouse

trials has now seen it extended to field trials (Adenle et al., 2012; Caldwell, 2008). Consumer demand for omega-3 fatty acids is growing as research continues to show that diets rich in omega-3 fatty acids are important for maintaining heart health. Omega-3 can be found at high levels in fish but many consumers are unable to eat this animal (e.g., vegetarians and vegans). Fish are also expensive to incorporate in the diet on a regular basis. To overcome this obstacle, Dow and Monsanto attempted to (and were successful in) developing a canola seed that produced the omega-3 fatty acid, docosahexaenoic acid. This is a health solution that can be offered by plant biotechnology to resolve the concerns of micronutrient deficiencies.

4 Fulfill Food Needs by Increasing Food Production

Billions of people in the developing world are undernourished and survive on minimal food rations. Furthermore, millions of people do not have secure access to food (World Bank, 2000). Among these, small-scale agriculture land holder farmers depend entirely on their own food for subsistence and to make a living. As they struggle for survival, how can they afford to irrigate their crops or purchase herbicides and pesticides for crop production? The world's population is predicted to double over the next 40 years. Based on this calculation it will be tough to meet the increased food demand without increasing food production (Rosegrant et al., 2001). Biotic and abiotic stresses are the major barriers to increasing food production and GM plant technology is just one of a number of approaches that is being developed to combat these problems. Pest infestation is responsible for major yield losses (up to 90%) in agronomically-important crop plants including cash crops (Kissoudis et al., 2014; Sharma, 2001). These pests actively feed on various plants to enable their development and progression into the reproductive adult phase. Current strategies of insect control rely heavily on conventional chemical control, which results in the development of resistance to almost all insecticide groups. Progression is stuck between insects and humans: insects are trying to develop resistance against toxic compounds while humans are trying to develop new control strategies (Mithofer and Boland, 2012). Insecticide resistance or the evolution of insecticide resistance and its subsequent spread in insects is common (Hemingway, 2000). Many species have been heavily exposed to insecticides and resistant populations have been reported in more than 40 countries (in both greenhouses and open-field crops) (Forrester et al., 1993; Georgiou and Lagunes-Tejeda, 1989; Hemingway, 2000; McCaffery, 1998). Increased pesticide use is also harmful to the ecosystem as a whole. The relative harmlessness to mammals (including humans) together with their low persistence in the environment and their moderate ecological impact have led us to concentrate on various techniques that can confer them safe to ecology. Therefore the emphasis has been placed on biopesticide research and usage. Biotechnological methods of pest control are ecofriendly, sustainable, and are the need of the time (Dawkar et al., 2013); however, transgenic crops that produce *Bt* toxins offer safe and specific insect control (Tabashnik et al., 2005, 2008). Although *Bt* toxin is lethal to many insects, multiple scientific studies have found them to be harmless to wild mammals, birds,

pets, and humans and therefore *Bt* endotoxins may as well be considered as biopesticides (Sharma et al., 2010). Alongside increasing crop yields, *Bt* crops also require less pesticide use. Insect-resistant cotton has now been stacked with herbicide-resistance traits and is awaiting regulatory approval. However, as mentioned earlier, abiotic stresses, such as salinity, drought, and temperature are likely to be important in the future and severe losses occur due to the extremeness of these factors. Hence there is a need to implement new technologies to ensure crop survival against all type of stresses. Specifically, studies are underway to produce GM plants to increase crop yields or to directly improve nutritional content. A commercial virus-resistant papaya is one successful example (Gurr and Rushton, 2005). Other successful examples of GM crops include insect-resistant GM maize, *Bt* cotton, and a study demonstrating that expression of an enzyme in GM maize activates an oxidative signal cascade that confers cold, heat, and salinity tolerance (Shou et al., 2004).

5 Safety Issues

5.1 Potential Effects of GM Plants on Human Health and the Environment

GM crops permit improvements in food quality and the production of recombinant medicines; however, issues have arisen regarding their impact on human health and their effect on the environment. These issues are usually put forward by the general public or anti-GMO lobbyists (Fig. 12.2). At present, GM crops have not caused environmental issues, but it is not known whether they will pose a risk in the future. Therefore before GM plants are released into the environment it is essential to establish if any negative environmental impacts could be possible further down the line. The effect on biodiversity; the effect on nontargeted insects, spiders, and other animals; the spread of novel genes; the identification of compatible partners for crossing; the flow of genes and crop specific information; and gene transfer to the environment should all be considered. A threat to soil ecosystems, risk for aquatic life, and swapping one pest for another are also possible. Furthermore, ecosystems with high

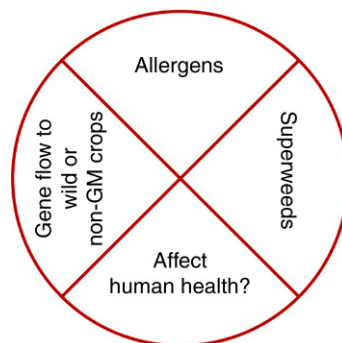


Figure 12.2: Defeats of GM Plants.

GM crops may lead to the emergence of new allergens, contaminate to wild landraces, can create superweeds and may affect to human health.

biodiversity must be kept intact and healthy in the face of disturbance and environmental change. Assessment of the overall health of an ecosystem should use biodiversity as a useful parameter. Therefore agricultural practices that use GM crops ought to maintain a balance in this respect. From an unbiased perspective we simply do not know whether GM crops are safe for human or animal consumption.

5.2 Allergy Concerns

Progress toward developing GM crops to enhance crop yield is essential to fulfill food needs. Although care has been taken regarding the safety of GM crops, our knowledge of their allergenicity is far from complete. Allergic reactions caused by GM crops has been one of the prominent concerns among the general population. Allergens may be produced through the addition of transgenic material and there is also a concern that the engineered or modified organism could produce endogenous known or unknown allergens. Various nuts, plants, and aquatic animals are known to cause allergies in certain individuals and these cause persistent and lifelong conditions. Milk, egg, soy, peanut, tree nuts, shellfish, fish, and wheat are the most common food allergies in humans. There is an enigma as to why certain individuals are affected and why the allergy is mainly restricted to childhood. Food intolerance or food allergy is to be understood through proper diagnosis as there is confusion between these terms. However, allergies against plant products are becoming a widespread problem and hence public awareness and concern about food allergens is growing. Therefore before a GM crop can be marketed it must pass several compulsory regulations. Exposure to an allergens in a sensitized person can lead to an allergic reaction, and further exposure can result in an adverse reaction, the most severe of which can result in death from anaphylaxis. There are two different types of food allergies: (1) caused by an overactive immunological response and (2) caused by dietary difficulties, such as lactose intolerance (i.e., an enzyme deficiency rather than an allergy). The enhanced immunological response is a true food allergy and can be severe. These allergens need to be regulated before being released as GM crops. Sometimes genetic components may also be responsible for food allergies in some families and modern diets containing plant-derived products can result in food allergies. The World Health Organization built regulations to define the allergenicity of proteins ([WHO, 1991, 2000](#)) but it is still difficult to predict with any accuracy. Multiple tests can be performed after the generation of new GM crops to determine allergenicity. As public awareness is growing about food allergens, the application or implementation of genetically engineered crops can be of serious concern.

6 Gene Flow and Landrace Contamination

Anxiety levels regarding environmental and ecological biosafety are alarmingly high following the development of GM crops. Determination of the long-term environmental impacts of GM crops is complicated and unpredictable and therefore implementing GM

crops is complex and difficult. Although GM crops have many benefits and will be helpful for resolving food security and quality issues, they have also aroused considerable biosafety concerns. One major and probable environmental threat is landrace contamination by gene flow to wild plants. Gene flow means the transfer of genetic material from one plant to another and this tends to occur during hybridization. It is possible that pollens from GM plants could contaminate non-GM plants eventually affecting local wildlife populations. Possible environmental consequences of this contamination could be a loss of genetic diversity among landraces and wild relatives. This may result in a loss of original vigor and fitness and potential destruction of the plant, hence affecting the ecosystem and human health. To avoid potential environmental consequences caused by the transgene, a systematic risk assessment is necessary under various conditions. To avoid gene flow in landraces, transgenes need to be taken into safekeeping using physical and biological approaches (i.e., terminator genes linked to the transgene).

Global dispute regarding landrace contamination arose after reports identified the presence of transgenic DNA in native plants. The loss of genetic diversity in crops will reduce their capacity to breed more productive and stress-resistant crop varieties. The adoption of GM crops by farmers due to their ability to produce more income and less expense may lead to a rapid decline in traditional crop varieties; for example, *Bt* cotton comprises more than 70% of the total cotton cultivation area in China and more than 40% of the total cotton cultivation area in India (James, 2006; Wu, 2007). There was reported the presence of transgenic DNA constructs in native maize landraces grown in the remote mountains of Oaxaca, Mexico (Quist and Chapela, 2001). A key concern is whether the transgene will have a deleterious effect on the diversity of maize landraces that these farmers cultivate. Mexico is considered to be one of the last reservoirs of genetic maize resources as Mexican farmers domesticated maize some 6000 years ago (Matsuoka et al., 2002; Piperno and Flannery, 2001; Sanchez et al., 2000). This diversity persists in today's traditional agricultural systems but the presence of a transgene in a native maize created a storm in Mexico and the rest of the world. However, further investigation of this study found flaws and a number of other groups disagreed with the conclusions (Kaplinsky et al., 2002; Metz and Fulterer, 2002). Replication of this work also failed to detect transgenes in wild Mexican maize (Ortiz-Garcia et al., 2005).

Many types of transgenic rice (*Oryza sativa*) will be ready for commercialization in the coming years, including varieties with higher yields, greater tolerance of biotic and abiotic stresses, increased resistance to herbicides, improved nutritional quality, and varieties containing novel pharmaceutical proteins (Lu and Snow, 2005). Additional transgenic rice varieties are also likely to be released for commercial production in the next 5–10 years. These are awaiting approval by regulatory agencies before they can be commercialized (Brookes and Barfoot, 2003; Jia et al., 2004) and ecological assessments of the risks and benefits must be performed before they can be released in the market. Moreover, this

information must be made publically available so that the consequences of transgenic rice can be fully understood. Understanding the anticipated effects of transgenic varieties on ecology, human health, and landraces is necessary and the long-term success of GM technology will be dependent on these aspects. Preventing the flow of genes between sexually compatible species is difficult hence effective strategies are needed to control it. In an open field it is impractical and impossible to control the spread of pollen and this makes the need for environmental studies all the more urgent. Extensive research in this area is a must and cannot be avoided. Without further investigation it will be impossible to conclude anything about the impact of gene flow. Taxonomists, cytologists, plant population ecologists, evolutionary biologists, weed ecologists, crop scientists, biochemists, biotechnologists, and molecular biologists need to work together to provide the answers regarding gene flow and landraces.

7 Swapping Insect Pests for Targeted Attacks

GM crops can occasionally affect harmless, nontarget species; for example, long-term exposure to pollen from GM insect-resistant maize causes adverse effects on the behavior and survival of the monarch butterfly. A few studies on European butterflies have been conducted, but these studies suggest that the animals would suffer from pesticide-producing GM crops. The environmental effects of *Bt* corn and cotton are still up for debate. The monarch butterfly is a species close to the heart of America and any harm to it (by mechanical or artificial obsession) could create a firestorm in America. A report from Cornell University on the effect of *Bt* maize on the monarch butterfly was seriously discussed by the agricultural industry, the US Department of Agriculture, the US Environmental Protection Agency, and elements of the North American academic community. Some other studies have supported the concept that the monarch butterfly is adversely affected following exposure to pollen from GM insect-resistant maize ([Cornell University, 1999](#); [Losey et al., 1999](#)). Studies on European butterflies have also been conducted in the same manner using *Bt*11 and MON810 varieties of maize. Even beneficial insects that are important for integrated pest management (i.e., green lacewings) have been adversely affected by GM *Bt* crops, such as green lacewings. However, the single-tier risk assessment has been widely criticized by scientists who call for a more holistic assessment. Green lacewings are reported to be affected by GM *Bt* crops, which are actually beneficial insects that play a role in controlling maize pests ([Obrist et al., 2007](#); [Harwood et al., 2005](#)). Furthermore honeybees too hampered by GM (*Bt*) crops ([Ramirez-Romero et al., 2008](#)). Although these crops have only affected beneficial insects and not higher animals, their effects are still considered to be important because they have ultimately affected the ecology. Soil ecosystems may also be adversely affected as the toxins from GM plants may enter into the soil ([Flores et al., 2005](#)) and residues from the soil may make their way into water sources, hence GM crops could pose a risk to aquatic life ([Rosi-Marshall et al., 2007](#)).

8 *BT Crop Resistance*

Detoxification occurs when toxins are modified by reduction, oxidation, and conjugation reactions, resulting in the excretion of modified toxin molecules. Insects employ one of these mechanisms to nullify the effects of toxin (Nishida, 2002; Price, 1997; Scott and Wen, 2001; Silva et al., 2001). During coevolution, plants and insects coped with their adverse surroundings and skillfully adapted. Expressing *Bt* Cry proteins in crops is currently the most commonly used and significant strategy for protecting against insect pests; however, several studies have reported the presence of *Bt* resistance in insects (Atsumi et al., 2012; Gahan et al., 2010). The ability of insects to adapt and develop resistance mechanisms threatens the continued success of *Bt* transgenic crops. The presence of resistance in targeted insect species has led to the assumption that toxins produced by GM crops will be harsher to beneficial insects. Hence researchers need to identify and develop new insecticides that will not be perilous for the environment.

9 *Superweeds*

The effect of herbicide-resistant crops will be reviewed in this chapter. When glyphosate-resistant crops were introduced there were no glyphosate-resistant weeds; however, the evolution of resistance to glyphosate led to the development of glyphosate-resistant weeds, which have dominated herbicide-resistant crops. Herbicide-resistant weeds are now known as “superweeds.” This provides an example of things to come and a close eye must be kept on all aspects of GM products before they reach the market. Without authentic mandatory safety testing these products should not be available for human consumption. If analyzed closely, the environmental impact of GM plants can also be seen. This raises the question of whether GM crops are really fulfilling their requirements.

10 *Bioethical and Social Issues of GM Crops*

Are GM crops really making a difference in global poverty and hunger? If not, why do we still have an interest in using GM crops? And if yes, why are poverty and hunger still issues? The main question asked by the general public is, “are there any harmful effects of GM crops on human beings or the environment?” Transforming genes from animals to plants or vice versa is not ethically or morally acceptable and so all of these aspects lead into moral, religious, and political disputes. There is no doubt that GMOs and plants have good intentions but notoriously bad reputations around the world. With the advancements in GM technology, civil society groups have been established that are concerned about the potential biosafety risks and ethical issues associated with GM crops. “Pro” and “anti” GMO lobbyists attempt to share their conceived views with the public and try to convince them of their beliefs. “Pros” seek to communicate the benefits of GM products while the

“antis” try to demonstrate that these benefits will be short lived while the adverse effects will harm humans and the ecology in the long run. Before GM crops can be released into the environment, there is a need to look into the ethical questions that adjoin society and ecology. Public reflections are always triggered by discussions in the media and thus public awareness is important. This may involve explaining the biotechnology behind GM to the public, which may go some way to answering their doubts. The views and opinions of the public cannot be dismissed and scientists should learn from them; however, at the same time, if researchers were to remain bound by public opinion there would be no possibility of producing medicines by genetic engineering. There is a big question about whether the issues raised by anti-GMO lobbyists are through annoyance or whether they are thinking rationally, morally, and ethically.

Public support for GM products has been exaggerated due to numerous ethical concerns over GM foods. As mentioned earlier “pro”- and “anti”-GMO campaigners have triggered controversy and regulations around GM foods due to their environmental concerns and the belief that these foods will contaminate the food chain and lead to new diseases and allergies. Ethical fears regarding GM crops is messing up the environment. Once GM crops are released into the environment it will not be possible to control them. People believe that even if we try to eliminate them, some part of the GMO will remain in the environment. Risks to the food web is a very real ethical concern of GM technology. There are reports that show that toxins produced by GM crops are harmful to nontargeted or beneficial organisms in the ecological chain. One incident in England was heavily debated by all parties. The issue involved the introduction of herbicide-resistant sugar beets, which was found to indirectly affect a native farm bird, the skylark ([Watkinson et al., 2000](#)). Introduction of this crop led to a reduced number of weeds and the skylarks, which rely on seeds from this weed, were indirectly affected. There was no way for these birds to find another type of food and in this manner many species, which are monotonous or monophagous, could become endangered.

Finally, environmentalist concluded that GM crops can affect biodiversity and hence are not good for the ecology or humans. There is no doubt that if weeds are left to grow they will compete with crop plants for the available nutrition and subsequently affect the crop yield; however, some weeds are important for natural farming. The aforementioned example is to understand the importance of biodiversity. The birds also survived on some pests, may consume them. These birds or animals can then prevent other pests on the farm. In the end, there is concern about whether GM crops can maintain biodiversity. At the moment it is thought that GM crops have a completely new ecological role, causing them to outcompete other species in the ecosystem. Crops that are engineered to be resistant to pests or herbicides can influence biodiversity because they may not only affect the target insect. Reduction in the population of an insect could harm biodiversity as it could be a source of food for another form of wildlife. Thinking about this issue from one point of view, instead of considering

many aspects of ecology, may be harmful to the environment, therefore respecting biodiversity and the environment is of utmost important.

11 If Anything is Wrong With GM Technology There is no Turning Back!

Several GM technologies have positive and negative effects. It is always better to understand all probable outputs so that an informed decision can be made to accept or reject the technology. The same is true for genetic engineering (GE) in agriculture. The issues raised with this technology are the potential risks to human health and the environment. To mitigate the impact of insect pests on agriculture, scientists have developed crops containing insecticidal *Bt* proteins. These *Bt* proteins kill insect pests but cause little or no harm to other organisms (including humans). However, their continuous use and large area of cultivation has lead to sporadic cases of *Bt* toxin resistance. This will ultimately lead to the development of superbugs, which are more difficult to control (Tabashnik et al., 2013). The second major problem with GM crops is that herbicide-tolerant crops are cross-pollinating with their wild cousins and creating herbicide-resistant weeds (Heap, 2014). The use of glyphosate to control weeds in herbicide-resistant fields could impair soil microbial communities, which will increase plant vulnerability to pathogens. It could also reduce the availability of certain soil minerals and micronutrients (Benbrook, 2012). During the development of GM plants, bacterial antibiotic resistance genes are often used in their construction, which becomes part of the plant genome. Cultivation of such GM plants could power the evolution of drug-resistant bacteria (Bennett et al., 2004).

DNA is composed of alternating sugar and phosphate molecules and different chemical bases (adenine, guanine, cytosine, and thymine). These four bases act as the genetic code and a specific sequence of these bases (which are bonded to each other by atoms of hydrogen) constitutes a gene. These four bases construct and define all life forms on Earth. If one of these bases was to change position (mutate) the damage could be phenomenal. In the human genome approximately 97% of all DNA has no known function.

Previous technologies (i.e., DDT) have been shown to have adverse effects in fish and fish-eating birds (i.e., eagles and ospreys) (Lear, 2009). DDT has since been banned but these types of concerns lead to questions about what harmful effects may be associated with the use or release of GMOs? The same question is also being asked by the public. This is not an easy question and being able to answer it requires an understanding of complex biological and ecological systems. To date, one cannot fully know the harms associated with GM crops. Some, but not all, GMOs may be harmful in the future by the sheer nature of their gene combinations and this must be critically studied; however, halting the use of this technology is not the answer to this question as multiple problems have been resolved using this technology.

12 Food Security

Food security is paramount and is dependent on a sufficient amount of food production and continuous availability. Food must also be nutritious and its composition must be understood (i.e., calories, proteins, fats, and carbohydrates). Food and nutrition security is associated with human health and economic and political stability. Food production will need to increase as the population of the world is ever increasing. As mentioned earlier, to date food security is totally dependent on infrastructure (e.g., the ability to deliver the food to appropriate places) and the political will to implement food plans and distribution food to those in need. It is a multifaceted issue that is ultimately related to the survival of mankind.

The main question is whether global food scarcity is due to the unavailability of food or reduced food production, or whether food is available in sufficient amounts but is not reaching the correct individuals. Once this issue is concluded we can then address whether to increase “food security now by GM crops or wait for research to assess risks?” To be able to achieve this it is necessary to review the approaches to agricultural development and the framework for agriculture and resource management at multiple interfaces. Furthermore, it is important to understand the reasons behind the crop yield losses (i.e., biotic or abiotic stress) and to keep up with these problems we need to critically think about whether transgenic crops are the solution or the evil.

The history of farming has shown that the long-term feasibility of farm landscapes depends upon the maintenance of functional diversity in soils, crop species, seed germplasm, trees, animals, and insects to maintain ecological balance and nutrient cycling. It was concluded that biodiversity is the foundation of agricultural sustainability (Weis, 2007). One cannot deny the importance of each and everything in the universe, however, in the race for empowerment, many techniques have relied on productivity and marketability without thinking of the undesirable implications during implementation. In this way we are progressing toward the genetic simplification of crops and finally to a monocultural farm system. The essence of this type of agricultural system (i.e., keeping at-risk biotic stresses at bay and coping with this situation) is that we remain reliant on synthetic pesticides that attack beneficial, as well as adverse pests. There are some exceptions (i.e., the GR), but almost all other remaining control strategies are associated with loss of biodiversity.

In this chapter we tried to review all aspects akin to biodiversity including wild species, monoculture systems, crop rotation, synthetic pesticides, the GR, and GM crops; their benefits and risks to human health and food security; and public opinion (Fig. 12.3). Scientists need to consider the opinions of anti-GMO lobbyists and environmentalists. Furthermore, politics will also play an imperative role in solving this issue. Finally, public education is important before these GM crops are released into the environment.

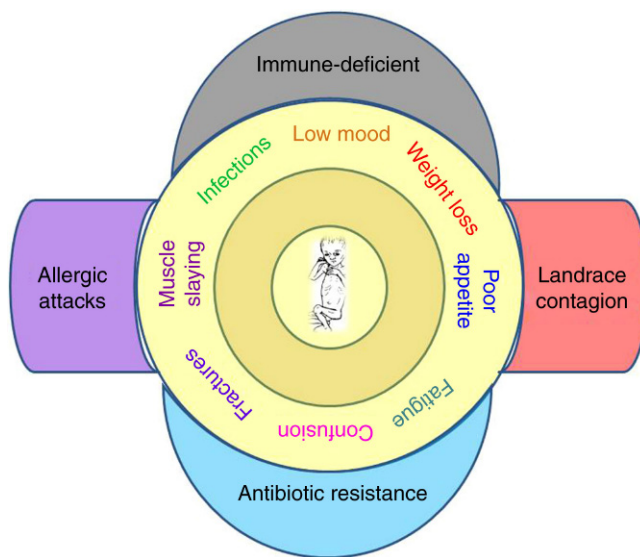


Figure 12.3: Positive and Negative Impacts of GM Crops.

GM crops have the ability to generate more nutritious varieties, possibly to the benefit the malnourished population. Environmental impact also has some promising effects; unfavorably there is lot of possibility to generate resistant weeds. Overall, adoption of GM crops increases farmers' income and it has given us drugs. On the other way round, it may lead to allergic attacks and antibiotic resistance in human beings.

The GR cannot be ignored when discussions about food security are taking place. The GR increased global agricultural production in the late 1960s by enhancing crop yields through the use of modern irrigation methods and fertilizers. Pesticides were already being used; however, improved crop varieties grew nicely. Many claimed that the GR had improved food security for a large number of people and this was shown to be true as the yield of wheat, rice, and maize was tremendously increased. In 1943 British-ruled India was severely affected by famine and the Bengal (now Bangladesh) famine was a world record food disaster. Approximately 4 million people in eastern India died of hunger that year. From then on "food security" was on the agenda of independent India. Soon after the implementation of the GR, India's status changed from a food-deficient country to one of the world's leading agricultural nations. The drastic improvement in yield was attributed to more land being used for farming, improved quality seeds (HYVs) being sown, and farmers attempting to take two crops per year. Although the GR was tremendously successful in certain parts of India (i.e., Punjab and Haryana), the rest of India did not reap the benefits. After a while the GR began to create problems, such as the adverse effects of pesticides and fertilizers on the environment and human health. Despite the effective implementation of the GR in India, the food demands have still not been met. The same scenario is also seen in other parts of the world. Taken this into account, it seems as though food security needs to be solved in other ways.

Food insecurity caused by an insufficient amount of food to meet the requirements is experienced in developing countries. The problem is long standing and it is not known for how long it will persist; however, the severity of the food shortage is becoming intense. Urgent assistance is needed for the affected population groups; however, it is not possible to keep up the same cut-off points for food supplements for everyone. The need for supplementation is dependent on the type of work carried out by individuals. Providing nutritious food in sufficient quantity to every human being is important. Hunger, poverty, malnutrition, and food deprivation are all connected. Instability in the climate has created problems for those with farm-based livelihoods, therefore the issue is how agriculture can adapt to climate change and keep up with the needs (food, fiber, and economic needs). Genetic engineered plants provide adaptable solutions. As mentioned earlier GM crops can thrive in the worst situations and most severe conditions. Pest resistance, salinity resistant, nutrition rich, drug production, paper and timber production, and HYVs can be accomplished using GM crops or organisms. Only the framework would need to be developed; however, this must be secure for the ecosystem. The issue remains that environmental activists are not ready to accept GM crops due to their adverse impacts in humans and the environment.

To try to keep up with the global food crises, the United States and India are trying to maintain efforts. In 2010 the US government launched the “feed the future” initiative to address global hunger and food insecurity. India also implemented a food agenda for all programs and the food security bill was passed in 2013. The US government launched a program to help poor people in developing countries achieve improved food security. It has been estimated that the global population is likely to reach 7–9 billion in the coming decades and therefore there is a need to increase food production; however, although the population will increase there cannot be an increase in the size of the cultivation area. Moreover, the worry is that agricultural productivity is likely to be hampered by climate change. Hence there needs to be a change in cropping patterns and an increased use of improved quality seeds while relying on limited land and a shortage of water. Food security is tightly connected to food availability hence increased food production is of utmost importance. GM crops are central to keeping up with the challenge of feeding a growing population. Implementation of GM crops will increase yields and eventually the income of farmers, acting as a sign of prosperity and reflecting the worldwide standard of living. Doubts about the use and regulation of GMOs has raised question about investing in more research. Hence start ups in this area are slowed down affecting from the developing of second-generation varieties. Once GM crop regulations are finalized (with surety of implementation) these crops will continue to address the challenges of food security. The only route down which to proceed in these circumstances is through genetic engineering. GM crops (i.e., rice, wheat, potatoes, fruits, and vegetables) are banned in most countries in the world; however, farmers’ yields in China and India increased due to the adoption of GM cotton. This scenario is also reflected in South Africa where farmers have benefitted financially and achieved an improved quality of life.

13 Conventional Breeding, Biopesticides, and GM Crops: What to Choose for Food Security?

Modifying plants to produce desired characteristics and increase production can be performed by plant breeding. There are many techniques to achieve breeding including propagation and molecular techniques. Plant breeding has been performed since the start of human civilization. It is easy and hence is commonly used by farmers, industries, government institutions, and universities. There are three methods of plant breeding: pure line selection, hybridization, and polyploidy. It is also possible to induce mutations via chemicals or radiation in breeding programs; however, these mutations cannot be controlled and therefore bring about the possibility of introducing undesirable characteristics. Plant breeding technologies were used to produce new crop varieties with desirable characteristics during the GR. In some areas it has been used to resolve the issue of food deprivation. Some biotechnological techniques are also used in traditional agriculture to achieve industrial applications. These techniques have shown promise in plant breeding and in improving the properties of economically important crops; however, the improved crop yields through the use of plant breeding technologies was only achieved for a very short period and was restricted to specific areas. For example, in India this technique was only successful in Punjab and Haryana. Furthermore, environments and growing conditions vary in different parts of the world and therefore it is not always possible to use the same seeds. Disease resistant, drought resistant, salinity resistant, herbicide resistant, and HYVs cannot be developed by breeding alone, therefore questions have been raised about the sustainability of this technology.

There are many polyphagous and agronomically important pests that infest economically significant plant species worldwide. Polyphagy, high mobility, fecundity, and facultative diapause are key physiological and ecological characteristics that facilitate survival of these pests even in unstable habitats. These pests are responsible for major yield losses in crop plants, such as cotton, sunflower, pulses, ornamentals, and cereals (Sharma, 2014; Dawkar et al., 2011). Current strategies for the control of insects have relied heavily on conventional chemicals, which has resulted in the development of pest resistance to almost all insecticide groups. The adaptation and detoxification mechanisms in these pests increases their resistance to nonhost plants and makes them difficult to manage in the field. Research has demonstrated the potential of insects to adapt to diverse phytochemicals and toxins (Bauer, 1995; Gill et al., 1992; Tabashnik, 1994). The number of independent origins of insecticide-resistance traits is important for studying the evolution of resistance and its subsequent spread. Synthetic insecticides are highly hazardous to human health and our ecosystem and therefore this has put a lot of pressure on research into and use of biopesticides. Biotechnological methods of pest control that are ecofriendly and sustainable are the urgently required (Giri et al., 2004); however, transgenic crops that produce *Bt* toxins can offer safe and specific control of pests although the evolution of resistance in pests has quashed their benefits (Tabashnik

et al., 2005, 2008). Therefore there is a need to focus attention on the polyphagous, herbivorous pests. Dissection of their resistance mechanisms and regulation of metabolic pathways will pave the way for developing new pest control strategies.

The neem tree (*Azadirachta indica*) has long been recognized for its unique properties both against insects and in improving human health. The most well-known phytochemical (azadirachtin and its derivatives belong to the limonoid group) insecticide in recent years was derived from the neem tree and was found within its seeds, leaves, and other plant parts (Musza et al., 1994). Although hundreds of plant-derived feeding deterrents have been discovered, none that are able to act solely as an antifeedant (unlike azadirachtin) have yet been exploited commercially (Warthen and Morgan, 1990). There is an implicit assumption that azadirachtin is a natural growth inhibitor that is able to influence insect hormones through interference with their neuroendocrine system (Rembold, 1989; Rembold et al., 1989). It also acts as a feeding deterrent for arthropods too. Most important is that azadirachtin is nontoxic to humans and therefore studies should be carried out to understand the mechanism and toxicity of azadirachtin against pests.

Progression is stuck between insect pests and humans: insects are attempting to evolve to develop resistance against toxic compounds while humans are trying to develop new control strategies (Mithofer and Boland, 2012). The primary means of controlling pests is by residual spraying with insecticides. Insecticide resistance, or the evolution of insecticide resistance and its subsequent spread in insects, is common (Hemingway, 2000). Many species have been heavily exposed to insecticides and resistant populations have been reported in more than 40 countries (Forrester et al., 1993; Georghiou and Lagunes-Tejeda, 1989; Hemingway, 2000; McCaffery, 1998). Increased use of pesticides is also harmful to the whole ecosystem. The relative harmlessness of pesticides to mammals (including humans) together with their low persistence in the environment and their moderate ecological impact have led us to concentrate on various formulations of extracts of plants (or individually purified compounds from plant parts) for pest control. The widespread use of synthetic insecticides incites fierce debate because of the risks to the environment and human health, as well as the potential for developing resistance in target insect populations. Therefore considerable research has been performed on biorational pesticides. Azadirachtin has attracted the most attention (Hu et al., 1996; Immaraju, 1998; Schmutterer, 1990) and has been reviewed extensively (Mordue, 1993; Mordue (Luntz) et al., 1998; Rong et al., 2000). Although azadirachtin interferes with the molting of insects, growth regulation, and sterilization, its cellular mechanism is poorly understood (Rembold et al., 1989). Its action as an antagonist of the molting hormone, and therefore its ability to cause alterations in the development of insects, as well as its effects on reproduction have been widely documented, especially in lepidoptera (Rembold, 1989). Moreover, in most cases it also disrupts their feeding habits (Hassanali and Bentley, 1987). In practice, neem offers promise in the fight against insecticide resistance because of the diverse modes of action of azadirachtin (Ascher, 1993). Therefore

biopesticides, such as azadirachtin could be a good option to target polyphagous pests. However, this only provides a solution for the control of pests and questions still remain as to how to fight against drought and salinity? How to develop nutritious food? How to achieve HYVs? How to create new crop varieties that can withstand the harshest climatic conditions?

14 Conclusions

Genetic engineering has intensely different aspects to the aforementioned techniques and hopefully it is seen as a superior versatile technology to keep up with the adverse situation. Conventional breeding can be used to produce new plant varieties without changing the genetic material and has therefore never received opposition from environmental activists. However, this is natural process and only specific characteristics can be introduced. This is an important biotechnique as it can be applied in medicine, agriculture, and genetics for the production of vaccines, drugs, and hormones. The main importance of GMOs is in the field and these will produce lifelong effects. It has been proved that many diseases can be treated with this technology; however, its opponents are not ready to accept GM crops due to their adverse effects (which are not yet proven but may be perilous in future). This chapter has attempted to put all aspects of GM crops before the audience and it now up to the general public to accept or reject these crops.

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Safety and Risk Assessment of Food From Genetically Engineered Crops and Animals: The Challenges

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1 Introduction

With the world population set to exceed 9.7 billion by the year 2050, human survival is becoming increasingly dependent on the development of new plant and animal breeding techniques, including modern biotechnology. The first food produced by modern biotechnology (genetic modification or genetic engineering) was the extended shelf life Flavr Savr tomato, which was commercialized by Calgene Inc. in 1994. The most important GE crops during 2015 were soybeans, maize, and canola amounting to a total global adoption rate of 175.3 million ha in 27 countries (James, 2015). The total global adoption of these crops has increased over the years and reached an accumulated peak of almost 2 billion ha (ca. 5 billion acres) in 2015 with developing countries planting more than industrial countries. These GE crops contain genes that confer herbicide tolerance (HT), insect resistance (IR), stacked traits (HT×IR), and drought tolerance (James, 2015). Other important commercialized crops include cotton and crops with low plantation areas include sugar beet, alfalfa, squash, papaya, tomato, potato, and sweet pepper. Insect resistant brinjal (also known as aubergine or eggplant) has been approved for commercialization and planted by farmers in Bangladesh; however, there are still disputes to be settled in court that were brought by Greenpeace. Crops have also been developed to accommodate consumer desires, such as enhanced nutritive value, antibrowning properties, high phytase content, high oleic acid content, and sugar control. High yields, pest and disease resistance, and tolerance to abiotic stresses are also engineered in GE crops (Flynn, 2003). The major GE crop growing countries are the USA, Brazil, Argentina, India, Canada, and China. A number of countries do not grow GE crops but may import GE grain and/or GE crop products. GE salmon (GE Aqua Advantage salmon) is the first GE animal and it was finally approved for human consumption by the United States in 2016. A study of the safety of GE food requires investigation into the

intended and unintended effects that could be harmful to humans and animals. The Codex Alimentarius guidelines provide a formal way of assessing GE crops and GE animals. It should be noted that some countries regulate the process (e.g., Europe) while others regulate the end product or the food commodities (e.g., the United States). The principles and basic requirements according to Codex are explained in this chapter. The new breeding technologies that go beyond the conventional transgenic methodologies are also briefly mentioned. Details of the Simplot potato, produced using gene silencing (RNAi), are also provided as an example to demonstrate how such crops would be assessed for safety. This chapter aims to give an overall introduction to the safety and risk assessment of food derived from GE crops and animals.

2 Safety and Risk Assessment of Food From GE Plants (Crops)

The Organization for Economic Coordination and Development (OECD) and the Codex Alimentarius Commission (Codex) were among the first international bodies to discuss the safety and risks of food from GE organisms and to prepare official guidelines to address this matter. These two bodies and the Cartagena Protocol on Biosafety (Table 13.1) were prominent in giving direction for the safety assessment of GE foods and addressing their risk to the environment. New and burning issues are on the agendas and guidance given by several of these international bodies is updated continuously.

The safety and risk assessment covers a number of phases including planning (e.g., problem formulation), studying the modification process (e.g., engineering), plant breeding (phenotype/agronomic analysis), and compositional analysis (assessing expressed protein(s) or other altered metabolites). Where enhanced nutritional value or reduced endogenous toxicity of the GE food is the objective, additional evaluation will be performed that would consider the benefits, as well as the risks.

2.1 Food Safety and Risks

The words safety and risk are often used interchangeably; however, for a clear understanding of the safety assessment of GE food a distinction should be made between risk and safety (which is the freedom from risk). The expression “GE food” is commonly used but is strictly speaking incorrect as GMOs are living organisms that can still grow and reproduce. The correct terminology is “food derived from GE organisms.” However, for simplicity the term GE food(s) has been used throughout this chapter.

2.1.1 Food safety

Wolt (2008) described the concept of safety as value laden that is understood within the “context of society, culture, politics, economics” (p.2) and one that can add trade. Safety profiles of food differ among cultures and societies. A given level of risk cannot be deemed uniformly

Table 13.1: International organizations involved in GE food safety.

Food and Agriculture Organization, World Health Organization, and Codex Alimentarius Commission

A body under the auspices of the FAO and the WHO are responsible for standards on food safety and quality. Codex Alimentarius Commission (Codex) prepared four standards for foods derived from modern biotechnology: (1) principles for the risk analysis of foods derived from modern biotechnology (Codex, 2009); (2) guidelines for the conduct of food safety assessment of foods derived from recombinant DNA plants, (Codex, 2009, pp. 7–33); (3) guidelines for the conduct of food safety assessment of foods produced using recombinant DNA microorganisms (Codex, 2009, pp. 35–55); and (4) guidelines for the conduct of food safety assessment of foods derived from recombinant DNA animals (Codex, 2009, pp. 57–76). These guidelines include sections on allergenicity, r-DNA plants modified for nutritional or health benefits, and the assessment in situations of low-level r-DNA plant material in food.

Organization for Economic Cooperation and Development

The Organization for Economic Cooperation and Development (OECD) is an intergovernmental organization representing 34 industrialized countries, which aims to coordinate and harmonize policies, discuss issues of mutual concern, and work together to respond to international problems. The OECD was one international body that contributed to the development of the principles and safety requirements of food from GMOs by means of a number of specialized committees and working groups composed of member country delegates. The consensus documents prepared by the OECD Task Force for the Safety of Novel Foods and Feeds (OECD, 2016a) are applied in the risk and safety assessment of food derived from GE organisms. The working group on Harmonization of Regulatory Oversight in Biotechnology is responsible for producing consensus documents relating to the biology of crop plants (OECD, 2016b) that are useful for the environmental risk assessment of GE crops.

World Trade Organization

Food safety is a fundamental requirement in the international trade of agricultural products. The Sanitary and Phytosanitary Agreement (SPS Agreement), a World Trade Agreement, is important for trading food products derived from GMOs. The SPS Agreement aims to ensure that SPS measures “do not represent unnecessary, arbitrary, or scientifically unjustifiable, or disguised restrictions on international trade, and recognize the sovereign right of members to provide the level of health protection they deem necessary” (WTO, 1995). The WTO recognizes measures applied by countries that are based on international standards, such as Codex; however, members are permitted to adopt measures that are more stringent than international ones, provided that they are “founded on scientific risk assessment, applied consistently, and are not more trade restrictive than is necessary” (Article 3 and Article 5). The Technical Barrier to Trade Agreement (TBT Agreement) covers technical measures and standards (i.e., labeling requirements) and aims to ensure that technical regulations do not create unnecessary obstacles to international trade (WTO, 1995).

The role of the WTO is illustrated by the dispute between the United States, Canada, and Argentina (as complainants) and European communities over the “de facto” moratorium on GMO approval from 1998 to 2004. The USA, Canada, and Argentina sought legal recourses at the WTO under a WTO SPS law based on unjustified and illegal denial of access to European markets (EC Biotech Products case), which resulted in financial losses to US farmers. The WTO found “in favor of the complainants in 2006, and based its final decision on the failure of the defendant to conduct “adequate” risk assessments, (SPS Article 5.1; Annex A (4)) by not taking the risk assessment techniques (protocols) of relevant international organizations into account” (WTO, 2006).

European Food Safety Authority

The EFSA was established in 2002 “to provide scientific advice on all aspects of food safety” to the European community. A number of expert panels (one of which is the panel for GE organisms), consisting of 21 independent scientific experts, assess new applications for the release of GE products in Europe. The EFSA takes on “selftasking” projects, such as the development of risk assessment guidelines “to stay in the forefront of new scientific developments” and to develop GM risk assessment approaches incorporating statistics, allergenicity, animal feeding trials, and postmarket environmental monitoring (European Food Safety Authority, 2015).

(Continued)

Table 13.1: International organizations involved in GE food safety. (cont.)*International Life Sciences Institute*

The ILSI is a nonprofit organization whose mission is to provide science that improves human health and wellbeing and safeguards the environment. It achieves this mission by fostering collaborations among experts from academia, government, and industry on conducting, gathering, summarizing, and disseminating science. It publishes on nutrition, toxicology, and GMO food safety (ILSI, 2012a,b).

Cartagena Protocol on Biosafety to the Convention on Biological Diversity

The Cartagena Protocol on Biosafety (CPB) is an international agreement of the United Nations to ensure the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology that may have adverse effects on biological diversity, taking into account risks to human health (CPB, 2000). See Table 13.3 for the definition of LMO.

acceptable as safe because of different norms of acceptability of risk in different societies (Wolt, 2008). Knowledge of safety has been gained over the course of many years through experience with traditional food, trial and error, epidemiology, and scientific knowledge. Safety assessments are normally essential components of the development of new food products whether formal regulatory approval is required. Food is considered “safe” if care has been taken during food production, processing, storage, handling, and preparation (Committee on Identifying, 2004). Humans tend to accept food as safe in many ways; for example, when it is produced by farmers, when it is sold at markets, or when it is prepared by people they trust.

2.1.2 History of safe use

The concept “history of safe use” is hard to define. Constable et al. (2007) described its characteristics under the headings “history,” “safe,” and “food” (Table 13.2). A similar description of “generally recognized as safe” (GRAS) is used for food or food additives. GRAS status may be obtained through a long history of safe consumption, scientific

Table 13.2: Key issues in determining the history of safety use (Constable et al., 2007).

History	Safe	Use
Correct identification	Composition (especially toxic, allergenic, metabolic, nutritional, and antinutritional components, as well as health compromising compounds)	Type or purpose (e.g., as a food, ingredient, supplement, or pharmaceutical)
Biology (origin and genetic diversity)	In silico tests (e.g., structural homology to known allergens or known toxins)	Preparation and processing
Length of use	In vitro tests (e.g., serum screening and digestibility tests)	Known precautions
Geographic/ demographic distribution of use	Animal studies (e.g., toxicology and nutrition studies)	Pattern of consumption (e.g., occasional, regular, or coadministration)
Details of use	Experience from human exposure	Intake (e.g., level, population exposure, mean/extremes)
Evidence of adverse effects	Clinical studies	
Reliability of data	Epidemiological evidence	

Source: Permission granted on September 19, 2016 by the author, Anne Constable anne.constable@rdls.nestle.com.

information about the nature of the substance, their customary or projected conditions of use, or the information generally available to scientists about the substances (USFDA, 2016a). There is no complete freedom from risk or absolute safety as many foods contain natural toxicants, antinutrients, and allergens that may cause harmful effects when present above accepted levels. Chronic exposure to small concentrations of plant toxins may only manifest later in life (e.g., cassava) (FAO, 2001). Food safety cannot be accepted as an absolute scientific concept; however, by comparing the composition of GE food with conventional food, progress can be made in the assessment of the safety of the GE food as long as the final conclusion is in comparison with the conventional counterpart.

2.1.3 Risk

Risk is understood in terms of the “likelihood of an undesired event” (Wolt and Peterson, 2000, p. 40) but zero risk does not exist (Querci et al., 2008). The probability of risk is quantitatively assessed by science-driven assessment consisting of four steps: hazard analysis, hazard characterization, exposure assessment, and risk characterization. Further explanation of these definitions is provided in Table 13.3.

2.1.4 Benefit

Scientific assessment of benefits is still in its infancy. Benefits may be characterized in a similar way to risks, but based on positive effects rather than hazards (Morris, 2011).

3 Approaches to Safety and Risk Assessment

There are a few important approaches for the safety and risk assessment of GE food that need to be explained.

3.1 The Targeted Approach

The concept of “substantial equivalence” was proposed by the OECD, discussed by working groups of the Food and Agriculture Organization and the World Health Organization (FAO/WHO), and incorporated into the Codex guidelines for GE food safety assessment (Codex, 2009; WHO, 1991, 1993, 2000). Criticism of the concept led to it being changed to “comparative safety assessment,” which is the starting point for risk and safety assessments (Kok et al., 2008). As has traditionally been the case with assessment of food safety, the approach for GE foods is to compare the modified food (e.g., raw materials, such as crop grains) with the conventional counterpart, focusing on the similarities and differences. This step in the risk assessment comprises the hazard identification step. This entails a comparative analysis at the molecular, agronomic/phenotypic levels, as well as compositional assessment. This concept is used in collaboration with the concept of “familiarity” in phenotypic analysis and “history of safe use” in compositional analysis. Equivalence is determined by statistically

Table 13.3: Definitions and explanations.

A cis gene: Is a natural gene coding for a (agricultural) trait from the crop plant itself or from a sexually compatible donor plant that can be used in conventional breeding. The gene belongs to the conventional breeder's gene pool.

A cisgenic plant: Contains no foreign genes (or) "if the donor gene and all of the transgene's regulatory sequences belong to the same crop species or belong to species that crossbreed with the host, the resulting crop is called "cisgenic." In the cisgenic technology, the cisgene must be an identical copy of the host's native gene cassette, including its regulatory sequences integrated in the host plant in the normal-sense orientation" (Sticklen, 2015).

Conventional breeding: Methods of crossing plants with desired characteristics to generate offspring combining those desirable characteristics. Methods described by this general term include selection, crossing, embryo rescue, somatic hybridization, somaclonal variation, mutation, and cell selection. Conventional breeding can involve both non-GE and GE plants (Steiner et al., 2013).

Event (also known as a GE single event, transgenic event, GE event, and single event): A single-locus insertion of recombinant DNA into the host genome regardless of the number of genes contained on the inserted piece of DNA (Steiner et al., 2013).

Genetic modification (genetic engineering): This is a technique used to alter genetic material of living organisms to make them capable of producing new substances or performing new functions. The result of genetic modification is a genetically modified organism (GMO or GM). This is a "transgenic" organism (UNEP, 1995), in which the genetic material has been altered in a way that does not occur naturally by mating or natural recombination.

Genetic modification techniques are: (1) in vitro nucleic acid techniques include recombinant DNA (rDNA) and the direct injection of nucleic acid into cells or organelles; or (2) fusion of cells beyond the taxonomic family that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection (CPB, 2000). rDNA is described as genetic DNA made by recombining fragments of DNA from different organisms.

GE Stack (also known as a GM breeding stack, stacked-event product, or combined event product): A plant produced by the conventional breeding of individual events that have been individually safety assessed (Steiner et al., 2013).

Intragenesis: Is the genetic modification of a recipient organism that leads to a combination of different gene fragments, which originate from donor organism(s) of the same or sexually compatible species. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism. Intragenesis involves the insertion of a reorganized, full or partial coding region of a gene frequently combined with another promoter or terminator from a gene of the same species or a crossable species (EFSA, 2012a,b). Intragenesis does not incorporate unknown or foreign DNA into a plant's genome (Rommens et al., 2007).

Phenotype: The set of observable characteristics of an individual resulting from the interaction of its genotype with the environment (http://www.oxforddictionaries.com/us/definition/american_english/phenotype).

Exposure assessment: The qualitative or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food, as well as exposure from other sources if relevant (Codex, 2016, p. 132).

Hazard characterization: The qualitative or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical, and physical agents, which may be present in food. For chemical agents a dose-response assessment should be performed. For biological or physical agents, a dose-response assessment should be performed if the data are obtainable (Codex, 2016, p. 132).

Hazard identification: The identification of biological, chemical, and physical agents capable of causing adverse health effects, which may be present in a particular food or group of foods (Codex, 2016, p. 132).

Table 13.3: Definitions and explanations. (cont.)

Risk characterization: The qualitative or quantitative estimation (including attendant uncertainties) of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization, and exposure assessment (Codex, 2016, p. 132). T-DNA: DNA encoded on a plasmid of <i>Agrobacterium</i> that is transferred to the plant cell. Trait: A phenotypic character of an organism. Transgene: Refers to any recombinant DNA in an event (Steiner et al., 2013). Although initially used to indicate any gene that was introduced into a genome through transformation, it is currently often reserved for genes derived from a different family (Rommens et al., 2007). Variety: Is used here interchangeably with cultivar, which stands for a cultivated variety.

comparing the endpoints. This is particularly evident in compositional analysis but also applied to phenotypic assessments. Slight differences between the test substance and the comparator are not viewed as safety concerns if they are within the normal limits of variation for that crop. Any intended and unintended differences are further investigated. This is also called the “search light” approach in contrast to the “bucket” approach, which involves “filling a bucket” with a lot of information, not all of which is important in a risk assessment (Raybould, 2010).

3.2 A Nontarget Approach

3.2.1 Whole food assessment approach

The concept of “substantial equivalence” was seriously criticized as pseudoscience (Millstone et al., 1999) based on the argument that scientists are not yet able to reliably predict the biochemical or toxicological effects of a GE food from knowledge of its chemical composition. However, Miller (1999) refuted this view, stating that substantial equivalence is not intended to be a scientific formulation, but rather a conceptual tool, and that it neither specifies nor limits the amount of testing needed for a new food. There has subsequently been a trend to expand the range of nonspecific testing, including extensive animal testing, even in the absence of any identified hazard (Raybould and Poppy, 2012). This is in spite of the fact that toxicological assessment of animals fed on GE feed is not feasible because of the difficulties in testing using whole food (Schauzu, 2000), (Section 7.4). Whole foods are complex mixtures of compounds, which vary in terms of their composition and nutritional value. Only small amounts can be fed to animals because of their bulk and their effect on satiety. Adverse effects not directly related to the material itself can be induced because the nutritional value and balance of the diet may not be suitable for the animal under test. Therefore it is not easy to assess safety by feeding animals with whole food.

3.2.2 Profiling approach

The profiling approach is gaining more recognition as more research is invested in it. With profiling, data on the properties of the GE crop are collected in an untargeted manner. “Omics” technologies including transcriptomics (mRNA profiling), proteomics (protein profiling), or metabolomics (metabolites profiling) are examples of this approach. Data gathered may relate to the gross phenotype or the composition of key tissues (Ladics et al., 2015, p. 591). These data are compared against a suitable conventional crop to identify changes that may be harmful or beneficial. Although baseline data on the “normal genetic variation inherent in plants” are still scarce, “considerable literature on the natural variability” (Harrigan et al., 2007b) of the components of some crops does now exist. The OECD consensus documents provide a summary of the available data (Section 5.3). The approach cannot be used in a standalone manner at this stage and it should be borne in mind that unintended changes are not necessarily harmful (Chassy, 2002, p. 171S). The criticism is that harm cannot be discovered in data, and profiling will not collect data indicating the seriousness of harm. It is also difficult to interpret the effect of altered metabolism in the absence of a hypothesis (Raybould, 2010). Depending on the specific case, screening for unintended and potentially adverse effects using these new technologies may require more informative and cost-efficient analytical methods. These are thoughts for the future. As yet there are no analytical or statistical methods that can sufficiently determine whether the profile of a new variety differs meaningfully from the existing one (Chassy, 2010; Davies, 2010; Harrigan and Chassy, 2012; Ladics et al., 2015).

3.3 Hierarchical, Multistep, Risk-Based, and Weight-of-Evidence-Based Approach

This approach is recommended by the US FDA for the safety and risk assessment of GE animals. The approach is hierarchical, multistep, risk based, and weight-of-evidence based. The approach is hierarchical as it “does not rely on a single “critical” study but rather on the cumulative weight-of-evidence provided by all of the steps in the review.” It is also event based. “Animals derived from different events even if they are based on a previously approved construct would require separate evaluations” (FD&C Act, 21 U.S.C. para 321(g)(1)(C)]. p.2).

4 Problem Formulation

A multistep framework for risk assessment has been proposed by the International Centre for Genetic Engineering and Biotechnology (Tepfer et al., 2013). It utilizes problem formulation as the first step of the risk assessment. Problem formulation is about framing questions that are relevant to the risk assessment and coming up with a plan to answer them. It involves outlining potential risks (leading to the creation of risk hypotheses) and then determining if the risks are plausible (e.g., testing and accepting or rejecting the risk hypotheses). The intention is to focus on what could go wrong as opposed to what might change. This

concept has been applied to many fields of research and in particular the environmental risk assessment is now applied to food safety and risk assessments. The steps followed include: (1) identifying a country's protection goals in the context of the regulatory framework goals (e.g., consumption of GE food) should not lead to adverse health effects in humans and animals (the pathway to harm), (2) gathering relevant information (e.g., the modification process, phenotypic/agronomic data, and compositional data), which may indicate the likelihood of harm occurring, (3) conducting an "initial" risk characterization in accordance with the Codex guideline, and (4) deciding whether it is fit for purpose; if the answer is "yes" then conclusions can be made and if the answer is "no" then an analysis plan is not developed.

The initial risk characterization in problem formulation would be the "targeted approach" according to Codex. Should there be any intended or possible unintended effects these would be further analyzed by means of risk hypotheses.

One disadvantage of this approach is that it only focuses on risks and problems and does not take into account the benefits (i.e., improved nutritional value). This only occurs as a secondary step.

5 Procedures for the Initial Risk and Safety Assessment of GE Plants Described by Codex Alimentarius

As described before, the risk and safety assessment of GE food follows the "Working principles for risk analysis" of Codex Alimentarius (Codex, 2009) (Table 13.1). The framework of GE food safety assessment follows a stepwise process. It starts with descriptions of the host plant used as food, the donor organism(s), the GE plant, the genetic modification, and the molecular characterization. This is followed by a phenotypic assessment (not included in the Codex guidelines) and a compositional analysis. The three comparative activities: molecular characterization, phenotypic/agronomic analysis, and compositional analysis, provide the data for the determination of substantial equivalence (Kuiper et al., 2001) and form the hazard identification step of the risk assessment. The assessment of the intended and unintended effects contributes to the hazard characterization. This is followed by exposure assessment and risk characterization.

5.1 Molecular Characterization

A definition of genetic modification and a description of the techniques are given in Table 13.3.

The characterization of the molecular changes resulting from the modification procedure is included in the risk assessment to obtain an understanding of the genetic modification, to determine whether the modification was as intended, and to determine whether any unintended disruptions occurred (OECD, 2010). The assessment includes:

- Critical assessment of the origin of the transgene (e.g., the bacterium, *Bacillus thuringiensis*) and the intended mode of action of the transgene product (Bravo et al., 2007; Pigott and Ellar, 2007).
- A description of the transformation method, (e.g., *Agrobacterium*-mediated, recombination, or biolistic) (Beadle, 1978; Bravo et al., 2007; Chilton et al., 1977; Sanford et al., 1987).
- A description of any DNA sequences that could potentially be inserted into the plant genome (OECD, 2010).
- A description of the inserted DNA and the insertion site including any genetic rearrangements, deletions, or truncations that may have occurred as a consequence of the transformation (OECD, 2010).
- A description of the RNA and/or proteins expressed from the inserted DNA in various plant tissues and/or at different times during plant development, the inheritance and genetic stability of the inserted DNA, as well as the stability of expression of the introduced trait(s) (OECD, 2010).

5.2 Plant Breeding

A safety assessment of the results from plant breeding is not included in the Codex guidelines (or in the requirements of a number of countries) because it is common practice that applies to the development of all new varieties. However, the information and details of breeding practices are of interest for the risk assessment of GE crops. A definition of plant breeding is given in Table 13.3.

Thousands of years ago farmers became interested in improving wild plant species for domestic use, thus the agricultural practice of plant breeding was born and resulted in the evolution of agriculture and human societies. Today almost every crop plant is related to a wild species that occurred naturally in its center of origin; for example, maize apparently originated from a Mexican maize-like plant called teosinte (Beadle, 1978). A vast array of traits, such as improved yield, IR, drought tolerance, improved nutrient composition, enhanced palatability, and decreased toxicity were selected and maintained. It is interesting to note that of the most common changes that have occurred in domesticated crops, 66% have taken place in secondary metabolites (Meyer et al., 2012). A new dimension in the evolution of plant breeding is currently underway as new gene transfer techniques are developed.

A perspective on crop genome plasticity by Weber et al. (2012) described the dynamic nature of the genome and the relevance of transposons (jumping genes). It also provided a better description of the molecular changes that are associated with traditional plant breeding methods, such as mutations, deletions, insertions, and rearrangements (Weber et al., 2012). Plant breeding includes an intense breeding and selection process where off-types are

traditionally eliminated. Despite these effects only a few safety concerns from traditional plant breeding have ever been recorded.

Plant breeding studies are used in hazard identification, in comparative assessment, and in environmental risk assessment. The agronomic and phenotypic analysis comprises criteria that describe familiarity with the crop. The GE plants are grown alongside conventional counterparts at a range of locations representative of different environments. The protocol for these studies includes a statistical analytical plan. The endpoints are set according to best plant breeding practices (resulting from breeders' experiences) and are selected for their agronomic importance (particularly the effect of the intended performance of the trait, e.g., HT, pest resistance, or drought tolerance). Common endpoints include phenotypic characteristics of germination and seedling emergence, vegetative vigor, time of anthesis, plant height at maturity, time of maturity, pollen characteristics, and yield. Descriptions of the biology of specific crops are available from the OECD ([OECD, 2016a](#)). These documents are of a general nature and may be supplemented with regional information and varietal information. During the selection and development of particular transformation events those where adverse effects are noted are eliminated. Data from the agronomic and phenotypic trials are compared for equivalency with the nearest isolate. Where unintended differences are observed, further safety investigations may be warranted.

New trends in molecular biotechnology are noteworthy. Transgenic techniques are gradually being replaced by a broader range of molecular breeding techniques. In the future even more drastic changes (i.e., strategies based on synthetic biology) may be applied for the development of new organisms. More information on new molecular modification techniques is provided in [Section 9](#).

5.3 Compositional Analysis

The OECD accepts that long history of use is a reassuring and practical starting point for evaluating the safety of GE food. OECD is responsible for the development of consensus documents for food and feed compositional analysis and these documents provide details of the compositional makeup of the conventional counterparts ([OECD, 2016b](#)).

The compositional analysis follows a targeted approach, as only components that are characteristic of the crop plants are included in the comparison. The comparison includes components of forage, whole grain and/or processed fractions of the crop. Components may include proximates (i.e., protein, fat, ash, carbohydrates, and moisture), fiber, minerals, amino acids, fatty acids, vitamins, toxicants, antinutrients, endogenous allergens, and secondary metabolites. The members of OECD have agreed on the crop components that are of importance for a particular food. Proof of compositional equivalence provides an assurance of safety because of the safe history of use of these crops. A case-by-case assessment methodology is followed for each crop and new trait that it expresses.

The results of a large number of compositional comparative studies have been published and the results emphasize that if a GE crop and its non-GE comparator have a similar composition this is likely to indicate that the GE crop is as safe as its conventional counterpart; however, hazards may be associated with the observed differences. The components that have concentrations outside of the range of equivalency are assessed further. In cases where the composition has been intentionally modified (e.g., increased levels of procarotenoid B), evaluation for acceptable nutritional values is performed.

5.4 Natural Variation of Food Component Concentrations

A risk assessment requires more than a statistical analysis between the concentrations of the components in the engineered crop and the control. Genetic–environmental interactions and farming practices mean that natural variation can significantly contribute to the understanding of possible risk. Therefore, the characteristic components of the GE crop, its comparator, and a number of conventional non-GE varieties or hybrids are normally included in the same field trials. The trials are executed in three to eight areas that are representative of different environmental conditions. A broader picture of the variation is acquired from information in recognized databases and publications, such as the OECD consensus documents and the International Life Sciences Institute (ILSI) crop composition database ([ILSI, 2012a](#)). Concentration ranges collected for more than 20 years are documented in these databases. The effects of multiple germ-plasms, diverse GE graphical areas, and seasonal planting on the stability of the inserted gene(s) and the compositional profiles of GE maize and GE soybean varieties are available; for example, soybean phytoestrogen, vitamin E, and the fatty acid composition ratio seem to be very sensitive to these “natural” influences ([Berman et al., 2010, 2011](#); [Frank et al., 2012](#); [Harrigan and Harrison, 2012](#); [Harrigan et al., 2007a, 2010](#); [Herman and Price, 2013](#); [Zhou et al., 2011a,b](#)). These changes highlight that any difference between a GE crop and its near isoline comparator needs to be considered within the broader context of crop variation.

6 The Novel Protein(s)

The presence of a novel protein introduced by the modification is an intended effect. Harm caused by this protein could be due to toxicity or allergenicity, therefore two hypotheses have been formulated:

1. The protein expressed by the introduced gene will not cause toxic effects.
2. The protein expressed by the introduced gene will not cause allergenic effects.

6.1 Toxicity

Toxicity testing starts with a bioinformatic comparison between the introduced protein and known protein toxins and antinutrients. In many cases the introduced protein will have a

history of safe use. Provided that no sequence or structural homology with toxins is identified, then the next step is often an acute oral toxicity study in mice or rats using the isolated protein (normally expressed in a bacterial system to generate sufficient quantities of the protein). This is used to determine the maximum toxic dose. In toxicology studies this test is included to determine the LD₅₀. A single dose is normally given by gavage into the stomach, with at least five animals at each level of administration. It should be noted that the value of this type of test has been questioned.

Since cooking is one of the most common processing methods applied to food, the introduced protein would normally be tested for heat lability. If the protein is heat labile and rapidly digested in simulated gastric fluid it is deemed unlikely to be toxic.

It may be necessary to carry out a 28- or 90-day repeated-dose rodent toxicity test according to the OECD guidelines. Although this is not deemed necessary unless specific concerns have been identified, the European Commission currently demands a 90-day subchronic feeding trial as a standard procedure (Section 7.4).

6.2 Allergenicity

Food allergies are adverse immunological reactions to an otherwise harmless food or food component; true food allergies may involve several types of immunological response. While the most common food allergens have been identified and characterized, others still remain unknown. There are 1313 known or putative allergens, which amounts to a very small percentage of all food proteins. Most proteins that are capable of sensitizing people are present at concentrations of 1% or more.

In 2001 the FAO developed a decision tree to assess the allergenicity of GE food (FAO, 2001). This was followed by Codex guidelines that used a weight-of-evidence approach in 2003, which is now commonly adopted (included in CAC, 2009). Further recommendations were made by Goodman et al. (2008) and Goodman and Tetteh (2011).

The following tests are frequently carried out based on these documents:

- Bioinformatics comparison of 80 amino acid length sequences with sequences of known allergens. Tests for short amino acid matches (usually 8-mer) may also be carried out but carry the risk of false positives.
- If structural similarity with known allergens is found, screening with serum from individuals with known allergies should be conducted. The allergen database often consulted is <http://www.allergenonline.com>.
- Pepsin digestibility should be tested as resistance to pepsin digestion is a common risk factor for allergenicity; however, some studies have shown that there is no correlation between in vitro digestibility and protein allergenicity (Herman et al., 2007).

6.3 Endogenous Allergens and Toxicants

The possibility of a significant increase in endogenous allergens and toxicants in a GE crop is stated in the Codex guidelines and is considered by many regulatory authorities. [Weber et al. \(2012\)](#) considered it unlikely that completely new toxicants would be formed. Should any changes occur that fall within the expected concentrations of the existing natural allergens and toxicants, the comparative compositional analysis (following the OECD consensus documents) includes natural plant toxicants and so provides a method for the detection of increased natural toxicant expression. While the theoretical possibility of increased expression of endogenous allergens is a scientifically interesting question, individuals allergic to a specific food must avoid consuming both conventional and GE varieties ([Panda et al., 2013](#)). The analysis of allergens from the most commonly occurring allergenic crops have been included in the requirements of some countries (e.g., EFSA). Of the eight identified major allergens (soybean, groundnuts, cow's milk, tree nuts, wheat, fish, and crustaceans), the focus has specifically been on soybean allergens as these are the most prevalent ([Bush and Hefle, 1996](#); [Hefle et al., 1996](#)). Challenging questions regarding the possible unintended effects from allergenic plants have been asked by several authors ([Goodman et al., 2008](#); [Jansen van Rijssen et al., 2015](#))

6.4 Antibiotic Marker Genes

Antibiotic marker genes were included in the first generation of GE products to serve as a selection method for developers. These have since then replaced and are no longer used because of the concerns regarding the development of antibiotic resistance in the treatment of human and animal infections. However, some events that were approved early on do still contain these marker genes ([EFSA, 2009](#)).

7 Animal Studies

7.1 Nutrition Evaluation

The compositional analysis provides the elements needed for a nutritional evaluation. Wholesomeness studies with animals are often included in the assessment to complement compositional–nutritional evaluations. The objective is to assess the nutritional value of the GE crop and its dietary suitability for animals; however, it is unlikely that such studies would reveal unintended minor compositional changes that have gone undetected in the targeted analysis because of their lack of sensitivity. Wholesomeness studies should not be confused with toxicological studies as their purposes differ. The requirements for wholesomeness studies are critical to produce results that are reliable. Wholesomeness studies are designed to assess input traits (e.g., diseases, IR, and HT) and output traits

(e.g., enhanced nutritional values and reduced toxin levels). Poultry, lactating cows, or goats are normally used in these studies, but other food-producing animals, such as pigs are also considered depending on the crop to be investigated. Chickens are popular choices as they are sensitive to detect changes in nutritional values.

The commonly used parameters to measure performance include increased live weight and food intake, carcass yield, and absolute and relative weights of body parameters (i.e., breast, wings, and drums). Moisture, protein, and fat content of certain tissues (e.g., breast) could also be included (ILSI, 2003; [Taylor et al., 2007](#)).

7.2 GE “Output Traits”

The assessment of output traits (e.g., enhanced nutritional value) is important for both human and animal consumption. GE crops are predominantly designed for human consumption although these products may end up in the animal food chain. The research teams and regulators must decide for which animal species the nutritionally-enhanced GE crop is suitable. If the nutritionally-enhanced crop is designed for animal consumption, the formulation will require expert input. This topic will not be discussed further in this chapter.

7.3 Quality Control and Quality Assurance

Quality control and quality assurance are important and apply to both toxicological and long-term feeding studies. Some studies have been published without the critical application of quality control, which is essential for reliable statistical analysis. It is important to follow international sampling procedures for grain, green chopped forage, pasture, and mixed diets to ensure that the composition of nutrients and antinutrients in the diets of controls is identical to that in the test sample. Following these guidelines can also help to ensure that diets are in accordance with the species requirements at each stage in the growth cycle of the animal, that contaminants, such as mycotoxins and pesticides are managed, and to ensure that diets for ruminants differ from those of monogastric animals. A minimum number of replications (blocks) needed to detect a treatment difference at $P < 0.05$ should be included as suggested by ILSI ([Cromwell et al., 2003](#)). These precautions must be followed to minimize any variability in the results. The results should ideally be interpreted by scientists with experience in animal feeding ([Cromwell et al., 2003](#); [ILSI, 2007](#)).

7.4 Long-Term Animal Feeding Studies

Long-term subchronic toxicity feeding studies involves exposing laboratory animals (i.e., rats, mice, and dogs) to feed containing a range of concentration of the GE grain for 90 days. Results are then compared with control animals fed non-GE grain. The requirements of the

protocols are based on the OECD guidelines for the toxicological assessment of chemicals (OECD guidelines for testing of chemicals). Endpoints in these studies may include weight gain, feed intake, hematology, enzymes analysis, gross pathology, and histopathological analysis. The “no observed adverse effect level” is determined from such studies. “Omics” analyses could be included but must be based on clear-cut questions (GRACE, 2015). The European Union (EU) stipulates that these studies should be performed for all single trait GE crops. When such studies are submitted to the EU they are often included in the packages submitted to other countries as well. The GRACE project (funded by the European Union framework 7 program) was initiated in part because of concerns over the value of long-term feeding studies for GE crops as it had been suggested that other *in vitro* and *in silico* methods may be of equal value. Comprehensive 90 day feeding studies with maize MON810 were performed by GRACE but the conclusions that the scientists came to were in line with the original conclusions, which were that there were no safety concerns. The results of the GRACE project confirmed that, if there is no trigger from the preceding analyses, 90-day feeding studies do not add relevant information to the risk assessment (Zeljenková et al., 2014). The GRACE project also carried out a 1 year feeding study with MON810 and concluded that it did not provide information in addition to that provided by the 90 day study (GRACE, 2015).

Herman and Ekmay (2014) discussed the options where whole food animal feeding studies may be justified. The same strict requirements for designing and executing wholesomeness studies to meet good practice guidelines are required for toxicological feeding studies.

7.5 Limitations of Animal Feeding Studies

There is no international consensus on the need for animal feeding studies. Scientists, such as Kuiper et al. (2013) and Bartholomaeus et al. (2013) disputed the value of such studies, especially as a permanent requirement. Arguments that are often heard to motivate the inclusion of long-term animal feeding studies have been framed as a testable hypothesis by Bartholomaeus et al. (2013) and are summarized by Bartholomaeus et al. (2013, p. 4) “(1) commercial production of GM (GE) crops can and does produce unintended, unexpected, and unpredictable compositional effects unrelated to both the parent line of the crop and to the inserted transgene; (2) the unintended, unexpected, compositional effects resulting from the production of GE crop varieties are potentially of toxicological significance; (3) compositional analysis of new GE varieties is insufficiently sensitive to reliably detect these differences at levels of toxicological significance; (4) toxicity studies on whole feed are capable of detecting toxicologically significant differences that would be missed by agronomic and compositional analysis.” In reviewing a range of data from feeding trials Bartholomaeus et al. (2013, p. 4) came to the conclusion that “in fact they did not add value, were unnecessary, and scientifically unjustifiable.”

Arguments that are raised by [Kuiper et al. \(2013, pp. 781–784\)](#) against any animal feeding studies (other than case-by-case) are based on the comparative “robustness” of the agronomic, phenotypic, and compositional analysis; the wealth of data available for commercialized GE crops; and predictions of safety based on crop genetics. Toxicological studies have “low power” for detecting adverse effects; for example, a toxic substance produced as a consequence of a genetic modification would need to be present at concentrations of at least 4000 mg/kg in the animal diet (assuming median toxicity potential) to induce a toxic effect. Another argument against these studies is the increased use of animals in experimentation as a matter of animal welfare ([Kuiper et al., 2013](#)).

[Snell et al. \(2012\)](#) reviewed 90-day feeding studies, long-term feeding studies, and multigenerational studies of maize, soybean, and rice. They concluded that there were no health hazards and no differences in the comparison studies. [Bartholomaeus et al. \(2013\)](#) selected a strong search engine to identify past and present studies that used animals. OECD guidelines are adapted for food studies and the 28- and 90-day studies were most commonly used ([OECD, 2016c](#)).

A few studies longer than 90 days were included (multigeneration and reproduction) and again no differences were found between the GE food and the control. A similar situation had previously arisen with irradiated food and animal studies failed to provide conclusive results; analytical chemistry proved to be the most robust methodology in this case.

[Bartholomaeus et al. \(2013\)](#) explained that the basic principles of toxicological study design were to have power to discriminate between true effects and differences due to sample variation. The interpretive power of the protocol design must be able to distinguish between biological and toxicological significances versus statistical significance (since a change may be statistically significant but biologically irrelevant). This depends on a large number of considerations in the study design (e.g., range of parameters, pretreatment of baseline determination, dose escalation, age and sex of the animals, species, strain, and source of the animals). What is sometimes not included (or not considered important) is the extensive historical control data that is available for certain strains of experimental animals. A maximum tolerance dose (MTD) is normally used to set the maximum level in a dose range-finding study for 28 days for rodents. The possibility of determining a MTD value is limited with whole food because of the bulk and complexity of the test material. The “interpretation of statistical versus biological or toxicological significance of small differences” between the test substance and control is challenging ([Bartholomaeus et al., 2013](#), pp. 13–14). Design limitations render it almost impossible not to question the use of such long-term studies in rodents. The inherent low sensitivity of the 90-day whole food studies renders any result of highly questionable value. Toxicological long-term feeding studies with new chemicals are usually conducted with two rodent species (e.g., rat and mouse) and one nonrodent species (e.g., dog) but in these cases it is possible to achieve a higher dose than in the case of a GE crop.

7.6 Defective Studies

A few reports are quoted here to point out the pitfalls in study design and interpretation. Common deficiencies include inadequate experimental design (identity of test and control substances), inappropriate control crops or little information on controls, the presence of contaminants not being reported, nutritionally balanced diets not being confirmed, inadequate information on the study design and methods used, statistical analysis being either omitted or inappropriate, sample sizes being too small, and studies not being published in peer reviewed journals (Arjó et al., 2013; Ermakova, 2005; Evans and Pusztai, 1999; Séralini et al., 2007, 2012). Such studies have produced unreliable results and inappropriate conclusions: the paper from Séralini et al. (2012) was retracted. The ILSI best practice guidelines (ILSI, 2003; ILSI, 2007) provide detailed protocols for wholesomeness studies, which can be adapted for feeding studies with rodents and other animal species

8 Unintended Effects

The question of the occurrence of unintended effects is pertinent; however, the answer is complex as it covers all aspects of the risk assessment from molecular modifications; agronomic, phenotypic, and compositional assessments; and intended effects. Unintended effects refer to unintentional changes in contrast to intentional changes (Cellini et al., 2004).

Perspective could be obtained by looking at the existing knowledge from traditional plant breeding. Unintended effects have been reported from non-GE crop varieties at the point of commercialization, although this is rare. Prominent scientists are of the opinion that it is unlikely that completely new toxicants will be formed as a result of genetic modification. The observed changes in non-GE crops to date have only been seen at the same level as existing toxicants, including precursors or catabolic products (Steiner et al., 2013).

The explanation of several authors is summarized here. Unintended effects are expected as the comparator isoline and the near isoline can never be an absolute fit. It is a common misconception that the composition of a GE crop should be equal to that of its near-isogenic, nontransgenic comparator with the exception of the intended changes. However, it has been demonstrated that even for elite, nontransgenic, commercial varieties, single-plant selection can result in a marked variation in phenotype. This occurs because such lines are often not completely fixed for even the most basic traits (Herman and Price, 2013). Ladics et al. (2015) contended that it was unlikely that all methods of GE, non-GE, and conventional breeding would have an equal probability of unintended effects. They identified induced mutagenesis as the most genetically disruptive technique, and thus the most likely to display unintended phenotypic changes. Scientific results from “omics” studies comparing GE crops with non-GE crops have confirmed these thoughts.

Nontargeted techniques (e.g., genomics, transcriptomic profiling, proteomics, and metabolomics) are types of methodologies that have opened up possibilities for in-depth comparative studies to gain a better understanding of the genomic and environmental effects on the composition of crop plants. However, interpretation of the vast amount of information is a challenge, methodologies still need to be standardized and validated, and the techniques are currently qualitative and not fully reproducible ([Harrigan and Chassy, 2012](#)).

9 New Molecular Modification Techniques

9.1 New Plant Breeding Techniques

A collection of new plant breeding techniques that go beyond the conventional concept of genetic modification are cisgenesis/intragenesis, oligonucleotide directed mutagenesis, reverse breeding, RNA-dependent DNA methylation, site-directed nucleases (e.g., zinc-finger nucleases), agro-infiltration, and grafting on GE rootstock ([Lusser et al., 2011](#), [EFSA, 2012a](#)). These have been followed by other genome editing techniques as described later.

Transfer of a gene (cisgenesis) or DNA fragment (intragenesis) between organisms of the same species or from a cross-compatible species differs from transgenesis in that it can be conceived as a process that could occur naturally. Whereas cisgenesis may lead to a new organism that is indistinguishable from a conventional cross, intragenesis may produce an organism that is not obtainable by conventional crosses. Crops modified by cisgenesis or intragenesis should be free of markers and any vector backbone so that only DNA from the compatible species is introduced.

Programmable nucleases enable targeted genetic modifications by inducing site-specific DNA cleavage in the genome and DNA modification during repair of the broken sites. This leads to targeted genetic modifications including gene disruption, insertion or deletion of new genes or small sequences (known as indels), changes in specific bases, and chromosomal rearrangements. Zinc-finger nucleases and transcription activator-like effector nucleases were the first tools used for gene editing. These techniques enable mutations to be introduced into any gene, knockout of gene function, creation of a modified protein product, or variation in the expression of the product, thus creating genetic diversity with precision and good efficiency. Genome editing techniques are not transgenic, as foreign genes are not introduced.

The preferred method of cleaving DNA and modifying the gene sequence is the CRISPR/Cas9 system. The method resulted from the detection of clustered regulatory interspaced short palindromic repeats in bacteria and DNA cleavage by the Cas9 nuclease. In 2015 the CRISPR/Cas9 system was described in a Nature article as “the biggest game changer to hit biology since PCR” ([Ledford, 2015](#)). CRISPR technology has been successfully applied to

edit the genomes in rice, wheat, maize, canola, orange, and livestock. It has the potential to eliminate food toxicants and rapidly improve livestock breeding, among other applications.

Genome editing techniques enable directed mutagenesis of specific genes for silencing, increasing expression, or stacking of multiple genes at a single locus. These techniques are specific enough to target either single or multiple copies of a particular gene. They offer the potential for metabolic engineering to increase the production of valuable plant products by upregulating or silencing specific pathway genes, cooperative or competing pathway genes, or transcription factors (TFs) (Wilson and Roberts, 2014).

The extent to which these new genome edited organisms will be classified as GE organisms and subjected to regulation is not yet certain. The United States Department of Agriculture (USDA) has asserted that mushrooms edited using CRISPR/Cas9 to resist browning are not subject to regulation (Waltz, 2016) and the same applies to a new type of waxy corn (Brodwin, 2016). The Swedish Board of Agriculture has also determined that some plants edited with CRISPR/Cas9 fall outside the definition of a GE crop. The debate is ongoing particularly in the European Union (Sprink et al., 2016). The outcomes will depend on each country's legislation and definition of modern biotechnology and GEs. One argument supporting deregulation of many genome edited organisms is the fact that they can only be detected if information about the modification has been provided by the developer (Lusser et al., 2011), thereby, making enforcement almost impossible.

9.2 Gene Modulation

The products of GE crops that are developed by technologies that modify endogenous plant gene expression may be assessed slightly differently from GE crops developed by the well-described genetic modification procedures. New traits are sometimes dependent on small RNAs or regulator proteins such TFs, which modify the expression of endogenous plant genes. The potential of RNA interference (RNAi) to enhance or reduce the accumulation of specific metabolites in food and feed crops (e.g., to produce hypoallergenic plants) has been reviewed (Koch and Kogel, 2014). Proponents of these techniques (Parrot et al., 2010) suggest that safety assessments should be considered differently. RNA and TFs are GRAS and the genes encoding RNAi and TFs are important components of plant genomes. Crops produced using RNAi modification are not expected to produce heterologous proteins. Quantitative differences in endogenous plant components resulting from modulation of TFs can be assessed through agronomic performance and compositional analysis (Parrot et al., 2010; Petrick et al., 2013).

9.3 Critical Points in Food Safety Assessment

The innate potato (*Solanum tuberosum*) illustrates the critical points in food safety (Table 13.4 and Fig. 13.1).

Table 13.4: Case study: innate Russet Burbank potatoes (J.R. Simplot Company).

The J.R Simplot Company developed six potato varieties with lowered acrylamide potential and reduced browning and discoloration (known as black spot bruising) using a gene silencing technique (RNAi) (Fig. 13.1). These potato varieties and their progeny were deregulated by the Animal and Plant Health Inspection Service (APHIS) of the USDA as they were unlikely to pose a plant pest risk (USDA, 2015). The USFDA (2015) considered the safety and nutritional information to ensure that the regulatory and safety issues were resolved prior to commercial distribution. The intended effects of the genetic modifications were twofold. The first aim was to lower the levels of asparagine and reducing sugars in potatoes. Acrylamide is a carcinogen that is formed from a reaction between asparagine and reducing sugars when potatoes are cooked at a high temperature (i.e., baking or frying). Therefore, reducing the potential for acrylamide formation is a method for addressing a food safety concern. Simplot introduced DNA sequences containing inverted repeat segments of three genes to permit silencing of these genes. One segment was derived from a gene that codes for an enzyme that catalyzes asparagine biosynthesis, while the other two segments were partial promoter sequences of genes encoding proteins involved in converting phosphorylated starch to reducing sugars. The second aim was to reduce browning and this was achieved by introducing inverted repeat segments of a gene coding for polyphenol oxidase. The inverted repeat segments of these genes produce double-stranded RNAs that suppress transcript levels of the respective endogenous genes, resulting in reduced enzyme levels. The stability of the insert was confirmed over three propagation cycles. Transcript levels of the asparagine synthetase and polyphenol oxidase genes were reduced in all varieties; however, the effects on the transcript levels of the starch degrading enzymes were more variable.

Bioinformatics analysis of the junction sequences of the insertion did not identify any putative open reading frames (ORFs) that could encode any putative polypeptides with homology to known allergens or toxins. A single contiguous amino acid sequence was identified within the putative ORF, which was identical to a sequence from a known allergen (avocado); however, the significance of this finding was successfully contested as not relevant to allergenicity based on several scientific arguments.

Potato tuber composition was analyzed from multiple locations in the United States during the 2009–11 growing seasons. The tubers of the GE potato varieties, controls, and reference varieties were planted in a randomized complete block design consisting of up to five blocks per site. A total of 52 components were analyzed including proximates, total amino acids (17) including protein-bound amino acids and free amino acids, vitamins (i.e., B₆, B₃, and C), minerals (i.e., copper, magnesium, and potassium), total glycoalkaloids (potato specific), sucrose, and reducing sugars (i.e., fructose and glucose). Statistical analyses were performed and considered statistically different at $P \leq 0.05$. Additional statistical analyses were conducted for the glycoalkaloids and sugars. Statistical differences were found but were within the previously determined tolerance levels and combined literature ranges. The statistical differences were not considered biologically significant. The intended reductions in free asparagine and reducing sugars were found near the time of harvest in most events but the differences in reducing sugars were not sustained after 2–5 months storage. After heat processing the levels of acrylamide were reduced by 60% in the GE potatoes compared with controls and conventional potatoes. The conclusions were that there were no safety or nutritional concerns and that the GE potato was as safe and nutritious as the conventional counterpart (USFDA, 2015).



Figure 13.1: Innate Russet Burbank (left), Next to a Conventional Russet Burbank 30 min After Peeling. Reprinted with permission from J.R. Simplot Company.

10 GE Stacked Events

The term genetically modified stack refers to a plant in which two or more transgenic events have been combined by conventional breeding. An event is a single-locus insertion regardless of the number of genes contained on the inserted piece of DNA (Table 13.3 for the definition). The number of stacks has increased in recent years although most combinations are for IR and HT. Recently the trait drought tolerance has been added. The question arises as to how these stacks are evaluated? Concerns have arisen regarding genomic instability and the interactions between the products of combined events. In light of the plasticity of plant genomes and the knowledge from conventional breeding with a long history of safe use, Weber et al. (2012, p. 1849) came to the conclusion that the weight-of-evidence showed that “enhanced genetic instability from a transgene or from common sequence in two or more transgenes is unlikely.” “Even then, the probability that any genetic instability will lead to an altered protein or metabolic product that creates a biosafety issue is exceedingly small; this production of a GE stack does not measurably increase this probability” (Weber et al., 2012, p. 1849). The authors concluded that “there is no readily identifiable biological reason why genomic changes occurring in the breeding of a GE stack would be different in nature, scale, or frequency from those taking place in non-GE plants” (Kok et al., 2014; Weber et al., 2012, pp. 1849–1850). They also argued that evidence from studies of genetic stability, gene expression, and synergistic or antagonistic effects demonstrated that stacked events should not require any further assessment for food and feed safety than was required for single events. Note should be taken of these arguments as some countries still require compositional assessment of stacked events despite assessment of the individual events.

The metabolic pathways targeted in traits currently on the market are well known, and possible interactions between stacked traits can be “predicted based on assessment of the individual parent events” (Steiner et al., 2013, p. 1593). If single events have been rigorously assessed and no potential interactions have been identified then no targeted food/feed assessment for the GE stack is warranted (Kok et al., 2014; Steiner et al., 2013).

11 GE Animals

11.1 General

GE animals (e.g., rats and rabbits) have been produced since the early 1980s. The purposes of GE livestock (e.g., pigs, sheep, goats, cattle, chicken, and fish) include increased growth rate with reduced body fat, increased polyunsaturated fatty acid content, increased milk production, improved milk fat composition, increased wool growth, and improved disease resistance. Examples include pigs that synthesize phytase in their salivary glands; pigs, cattle, and goats that produce lysozyme (a natural antibiotic) in their milk to promote udder health;

and cattle that produce pharmaceuticals in their milk. The number of GE animals developed in laboratories is increasing but the number that are close to entering the market remains small (Forabosco et al., 2013; Hernandez-Gifford and Gifford, 2013).

The US Food and Drug Agency (USFDA, 2016b) currently divides GE animals into six broad classes based on the intended purpose of the genetic modification: (1) to enhance production or food quality traits (e.g., pigs with less environmentally deleterious wastes or faster growing fish); (2) to improve animal health (e.g., disease resistance); (3) to produce products intended for human therapeutic use (e.g., pharmaceutical products or tissues for transplantation; these GE animals are sometimes referred to as “biopharma” animals); (4) to enrich or enhance the animals’ interactions with humans (e.g., hypoallergenic pets); (5) to develop animal models for human diseases (e.g., pigs as models for cardiovascular diseases); and (6) to produce industrial or consumer products (e.g., fibers for multiple uses (USFDA, 2016b, pp. 3–4). Cloned animals are not the same as GE animals (transgenic), which is why they are not included in this classification (although both terms do relate to biotechnology techniques). GE animals discussed here contain heritable recombinant DNA (rDNA) constructs and the products of these animals could enter the food chain, whereas GE animals with nonheritable rDNA constructs are bred for use in gene therapy, for example, the USFDA regulates GE animals as a drug under the provisions of the Federal Food, Drug, and Cosmetic Act, since the intention is to affect the function of the animal [FD&C Act, 21 U.S.C. para 321(g) (1) (C)]. The approach is hierarchical, multistep, risk-based, and weight-of-evidence based (Section 3).

A comparative analysis requires molecular, phenotypic, and compositional comparisons with appropriate comparators. In this respect traditionally bred animals and their products can serve as references. Similarities and differences are identified and the outcome of this analysis can further structure the analysis (Codex, 2009, pp. 57–76; EFSA, 2012b; Jansen van Rijssen and Morris, 2015; USFDA, 2016a,b).

The health status of the comparators is of significant importance. Extensive analysis of their phenotypic characteristics including health and physiological parameters is performed (EFSA, 2012b) and this is followed by an assessment of the safety and risk of food and feed products from the GE animals. It is the requirements for comparators for animal species to obviously differ among food producing animal species. The European guidelines include honeybees used in agricultural practices but exclude insects and other invertebrates (EFSA, 2012b; Jansen van Rijssen and Morris, 2015).

Following characterization of the hazard, steps should be taken to estimate the likely exposure of humans and animals. This is followed by characterization of the risk including uncertainties, probability of occurrence, and severity of known or potential adverse health effects in a given population (EFSA, 2012b; Jansen van Rijssen and Morris, 2015).

The compositional components that are relevant to animal tissues, organs, fluids, and/or derived products are analyzed. The potential toxicity, bioactivity, and allergenicity of the gene products (and expressed proteins) and the metabolites of GE-derived food and feed are included in the safety assessment. The effect of processing on the characteristics of the food/feed is also important; therefore samples are taken at different stages in the productive life of the animal. A summary of the materials used in the analysis (EFSA, 2012b, p. 19) is provided here:

1. Animal body:
 - Mammal tissue: meat (e.g., muscle), body fat, blood, organs (e.g., kidney, liver, and spleen), and residue (e.g., bone meal).
 - Bird tissues: meat (e.g., breast and thigh muscle), abdominal fat, blood, organs (e.g., kidney, liver, and spleen), and residue body (e.g., bone meal).
 - Aquaculture: tissues (edible fraction, e.g., fillet) and residues (e.g., fish meal).
2. Samples for food/feed produced by GE animals:
 - Mammals (milk), birds (eggs), aquaculture (eggs), or insects (honey from bees).

The range of components to be assessed includes macro- and micronutrients (e.g., proteins, carbohydrates, lipids/fats, vitamins, and minerals), bioactive components (e.g., hormones and growth factors), key allergens, antinutritional compounds, and toxic compounds. These are selected on a case-by-case basis depending on the animal species.

11.2 *Atlantic Salmon*

11.2.1 *Molecular characterization*

Safety assessment of GE Atlantic salmon follows the steps described for GE animals. An example of a germline modification is seen in the first transgenic fish, AquAdvantage salmon (Fig. 13.2). This GE fish has been approved by the US APHIS for contained release after ~20 years of research. AquAdvantage salmon are produced by fertilizing eggs from a nontransgenic salmon with milt from a transgenic “neomale” homozygous for the rDNA construct expressing a growth hormone (GH). The “neomales” are females that are masculinized through the application of 17-methyltestosterone so that they produce milt instead of viable eggs, a process known as gynogeny. The offspring are all triploid, female, and sterile. The applicant claims that the transgenic salmon will reach maturity in about half the time of a conventional salmon. Characterization of the rDNA showed that the GE salmon contained a single, well-characterized copy of the construct inserted in a repeat region of the chromosomal DNA, not in a protein-coding region. The possibility of disruption to other genes was investigated and considered unlikely. There were no open reading frames flanking the insert and there were no sequence elements in the construct that contained coding regions derived from known toxicants, pathogens, oncogenes, tumor suppress genes, or sequences derived from transposable elements or retroviruses. Stability of the insert was confirmed over



Figure 13.2: Size Comparison of an AquAdvantage Salmon (Background) Versus a Nontransgenic Atlantic Salmon Sibling (Foreground) of the Same Age. Both Fish Reach the Same Size at Maturity but the Nontransgenic Salmon Will Take Twice as Long to Grow to the Mature Size. *Reprinted with permission from AquaBounty Technologies.*

seven generations. The FDA concluded that no hazards were identified during the molecular characterization of the construct (USFDA, 2015).

11.2.2 Phenotypic characterization of the GE animal

Phenotypic and health data of the GE fish line were generated for evaluation. A range of other sources were also considered for evaluation; not only controlled studies, but also historical records, other noncontrolled studies, and scientific literature. The evaluation included information on specific fresh water aquaculture practices (i.e., inland tanks, water specifications, hatchery and smolt production, fish stocking density, and disease status) and the role of culling practices in the selection of animals. This is necessary to confirm the potential role of practices to mask adverse outcomes. A limited number of effects were observed in the comparators, mostly in early life.

The studies included comparisons with diploid male and female salmon, as well as triploid female salmon of the same AquAdvantage salmon lineage. A number of altered phenotypic characteristics were reported in GH transgenic salmon relative to non-GE comparators, these included increased growth rate and associated changes (i.e., size, weight, and relative measurements) as would be expected from the introduction of this GH construct into the salmon. Increased frequency of skeletal malformations and increased prevalence of jaw erosions and multisystemic, focal inflammation were observed but within the limits of the non-GE animals. The induction of triploidy in salmon increased the risk of gill, fin, and heart abnormalities, and ectopic mineralization. The severity of these effects was generally minimal and was not expected

to have a significant consequence in a production setting. Reduction in growth characteristics due to the induction of triploidy is often reported in the literature; however, these reductions were compensated by the increased growth rates in GE salmon versus non-GE comparators. Some of the reported changes could potentially make these fishes less fit and less likely to survive if they were to escape from grow-out facilities. A phenotypic durability study showed that the phenotype did not change over multiple generations (USFDA, 2015).

The overall weight-of-evidence evaluation of the data indicated that there was no significant impact of the *opAFP-GHc2* construct on the overall health, morbidity, mortality, and incidence of malformations in GE salmon (USFDA, 2015).

11.2.3 Food composition

Substantial equivalency between AquAdvantage salmon and Atlantic salmon was confirmed through laboratory testing and by referencing the FDA regulatory fish encyclopedia. Possible changes in endogenous substances resulting from GH expression were subsequently investigated. Several studies investigated the levels of hormones in salmon tissues, which included insulin growth factor (IGF1), estradiol, testosterone, 11-ketotestosterone, thyroxin (T4), and triiodothyronine (T3), as GH enhances the conversion of T4 to T3. GH was below the limit of detection in all samples. The only difference was an increase in IGF1 concentration although the margin of exposure was well within the levels of exposure from other dietary sources. Several studies were conducted to determine the potential allergenicity of the newly expressed protein. Bioinformatic analysis showed that no amino acid sequence of 80 amino acids or more identified with known allergens by more than 35%, and no matches occurred for sequences of eight or more contiguous amino acids. No risk was envisaged. Tests for reactivity using serum from allergic donors did not reveal any significant differences between the GE salmon and conventional salmon. The conclusion was that this salmon posed no new allergenic risk.

Compositional analysis was conducted to determine the concentration levels of key nutrients in GE salmon compared with the comparators. Several groups of non-GE salmon with different genetic backgrounds, as well as the non-GE “near isoline,” were included in the comparative assessment. The fish were screened visually to assess their general health status and for traits relevant to commercial marketability (i.e., skin, fin conditions, color, markings, general body morphology, genotype, and ploidy analysis). The compositional analysis included proximates, minerals, vitamins, amino acids, and fatty acid content. Statistical analysis showed that all components were similar between the GE salmon and the control groups except for vitamin B6, which was higher in the GE salmon; however, the level was still well below the maximum recommended daily intake for this vitamin and it was not considered to be a food consumption hazard. Total fat content, fatty acids, and levels of omega-3 and omega-6 fatty acids were within the range of the controls and the ratio between omega-3 and omega-6 fatty acids was comparable with the controls (USFDA, 2015).

12 The Precautionary Approach (Principle)

A risk and safety assessment of GE food is a precautionary approach; however, because of uncertainty in the assessment (which is always present and forms an inherent part of a risk assessment) further precautionary considerations are taken into account by decision makers. It is generally accepted that precautionary measures should be taken where there are threats of serious or irreversible damage. A number of models exist for decision making based on assessment of risks in general. These rest upon axioms and assumptions that are not always valid in practice, such as perceptions of cultural differences. When faced with an unacceptable risk, scientific uncertainty, or public concern, decision makers have a duty to find answers.

13 Societal Decision Making

The risk analysis model provides a regulatory framework that includes risk communication. However, there are many challenges to effective communication, such as transparency and participation by stakeholders. If participation is not possible then scientific description of safety and risk assessments is a step in the right direction.

14 Benefit Assessment

Depending on the GE food, it may deliver various benefits, such as improved food safety, improved nutrition, or agricultural and environmental benefits. In addition, there are socioeconomic benefits relating to quality of life that have received little attention in risk management decisions. Higher yields from GE crops result in improved food availability and higher incomes for farmers, and drought tolerant crops hold great promise for maintaining food production in the face of climate change. How does, or can, the third world benefit from GE technology? Cotton production by smallholder farmers in the South African Makathini flats is an example of positive implementation of farming with GE cotton. The outstanding benefit that these small-scale farmers appreciate is the quality of life expressed as “more time with their family.” In Bangladesh, small-scale producers of IR GE brinjal have been able to increase yields and reduce pesticide applications by 80%–90%. In the Philippines, 270,000 smallholder maize farmers are able to derive improved household incomes from growing GE maize (Yorobe and Smale, 2012). These successes depend on markets and infrastructures to a large extent.

15 Conclusions

Traditional breeding techniques, such as mutation breeding, natural variation, and environmental effects often cause more inherent variability than genetic modification (Barros et al., 2010; Batista et al., 2008; Coll et al., 2009, 2010; Ricroch et al., 2011; Ricroch, 2013).

As a result, arguments for GE crops to be subject to less stringent requirements or exempt from regulations are heard frequently (Bartholomaeus et al., 2013; Herman and Price, 2013; Herman et al., 2007; Strauss and Sax, 2016). Perceived problems with the application of the precautionary principle mean that policy reforms are also called for (Durham et al., 2011). In this respect, Argentina decided to address the long delays and costly assessment requirements by developing a “construct-based” risk assessment framework for GE crops (Beker et al., 2016).

Increasing demands for safety testing of GE food are unwarranted and are making the barriers to entry impossibly high. This means that many beneficial new crops are not coming to market. And that the new breeding techniques represent a continuum from conventional breeding through mutation breeding to so-called “GE” so that there is no clear differentiation. Testing should therefore be based on specific scientific considerations that might give cause for concern rather than on blanket requirements that are not based on any specific risk hypothesis. Moreover benefits in terms of improved food safety and nutrition should be taken into account and balanced against the risks.

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Identification of Genetically Modified Foods

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1 Introduction

Modern biotechnology is trying to create new genetically modified (GM) foods that express interesting traits, such as insect resistance, herbicide tolerance, infectious-agent resistance, and resistance to environmental changes. The first commercial GM organism (GMO) was the Flavr Savr tomato (CGN-89564-2; “flavor saver”), which was produced by the Californian Company, Calgene. It was submitted to the United States Food and Drug Administration (FDA) in 1992 and on May 18, 1994, the FDA finished its evaluation of the “Flavr Savr tomato” and concluded that aminoglycoside 3'-phosphotransferase II could be used in the processing of new varieties of tomato, rapeseed oil, and cotton and that it was safe for food use (Meyer, 1995; Redenbaugh et al., 1992; Weasel, 2009). Food can be classified based on the presence of GMOs, as either: (1) food containing EU-approved GMOs (traits must be specified on the label); (2) food containing up to 1% of EU-approved GMOs; or (3) food of an organic nature whereby GMOs are not permitted (Maria, 2008). The traceability of GMOs is ensured using strategies and regulations based on molecular detection methods, which can be categorized as indirect or direct methods (Table 14.1). Among the numerous analytical methods that can be used for testing GMOs, real-time polymerase chain reaction (PCR) is considered as the “gold standard” (Brod et al., 2014; Mazzara et al., 2013). The main steps in identifying GMOs using analytical methods include: (1) detection (using screening methods, such as PCR to establish if a sample contains GMOs), (2) identification (using PCR based methods to establish if the detected GMO has been previously approved within the EU), and (3) quantification (using semiquantitative PCR, real-time PCR, and ELISA techniques to assess if the GMO meets the 1% threshold level, or the 0.3% or 0.5% level for seeds) (Maria, 2008).

Table 14.1: Types of methods use for detection of GMOs (Ahmed, 2002; Anon, 2014; Barbau-Piednoir et al., 2014; Basak et al., 2014; Brod et al., 2014; Buermans and den Dunnen, 2014; Chaouachi et al., 2014; Chen et al., 2016; Di et al., 2014; Faria de Melo et al., 2013; Fraiture et al., 2015; Hamels et al., 2009; Holck et al., 2010; Hoorfar et al., 2011; Kiddle et al., 2012; Kruse, 2004; Leimanis et al., 2006; Li et al., 2008, 2015; Liang et al., 2014; Lin et al., 2015; Marmiroli et al., 2008; Mazzara et al., 2013; Mbella et al., 2011; McKenzie et al., 2000; Morisset et al., 2008; Nadal et al., 2006; Nakaya et al., 2007; Navarro et al., 2015; Park et al., 2015; Randhawa et al., 2013; Rosa et al., 2016; Santiago-Felipe et al., 2014; Song et al., 2014; Tan et al., 2013; Tzu-Ming, 2002; Von Gotz, 2010; Wahler et al., 2013; Wei et al., 2016; Yeaman et al., 2016; Yelda Ozden, 2012; Žel et al., 2012).

Types of Methods		Methods	Features
Indirect methods (immunologic)	Phenotypic methods	Herbicide assays	Inexpensive and suitable for seeds
		ELISA	Detects proteins, faster, less expensive, and quantitative
		Luminex	Detects proteins and can be used as a screening method
		Immunoblot	Detect proteins, laborious, expensive, and sensitive
Direct methods (genetic)	DNA-based methods	Qualitative PCR	Sensitive, most precise in terms of detection limits, qualitative, and can detect known genes
		Quantitative real-time PCR	Very sensitive, rapid, expensive, detects known genes, most precise in terms of detection limits, and quantitative
		Loop-mediated isothermal amplification (LAMP)	Rapid, cheap, portable, and can detect known genes
		Microarrays	Expensive, detects known and unknown genes, and can be used for screening genes
		Microfluidic dynamic array	Detects known genes and can be used for the screening of genes
		Sanger sequencing	Expensive and detects unknown sequences
		NGS sequencing	Expensive, detects unknown genes/sequences, and can be used for the screening of genes

2 Sampling

Using the correct sampling method is vital for obtaining the correct results, irrespective of the analytical method used. Sources of error may be found in the sampling scheme, the testing plan, or in the accuracy and precision of the assay. Not all plants in a given crop will be contaminated with GMOs, therefore using a sample size, that is, overly small can

produce false-negative results. For example, some suggest that the size of a seed sample should be $\sim 1.5\text{--}3$ g, while others suggest that the number of seeds depends on the mass equivalent. The sample size depends on the method used and the detection limit of the GMO. The sampling scheme represents a method for collecting the field sample to assure that it is statistically representative of the whole crop. The sample analyzed in the laboratory is called the analytical sample and should be representative of the field sample. Crop sampling should be random, and a “w” walk assures a proper spread of sampling. Furthermore, plant locations should be recorded and marked in some way to permit future reanalysis. The sample volume taken for analysis depends on the suspected contamination level and the level of statistical assurance. For example, 100 plants should be analyzed to detect a 3% contamination level with a 95% confidence limit (CL), 300 plants are necessary to detect 1% contamination with a 95% CL, and 3000 plants are needed to detect 0.1% contamination with a 95% CL. The sample preparation is very important in terms of cross contamination. For example, the processing of large volumes of samples should be conducted in a controlled laboratory environment. Molecular biology methods are highly sensitive and air particles from the sample could lead to cross contamination and false-positive results. Equipment and instruments must also be cleaned after every run, as the presence of a few GMOs in the analyzer could cause positive results in the following samples (Anon, 2014; Kruse, 2004; Žel et al., 2012).

3 GMO Testing Methods

3.1 Indirect Methods

3.1.1 Phenotypic methods (herbicide bioassays)

Herbicide tolerance can be testing through phenotypic changes. In these tests non-GMOs and GMOs are cultivated on solid germination media in the presence of a specific herbicide to detect if the plants show distinct characteristics. Samples testing positive with this assay should be further analyzed and confirmed using more complex methods. These tests are inexpensive and allow companies to perform quality assurance testing for each batch of seeds (Faria de Melo et al., 2013; Li et al., 2008). Herbicide bioassays are widely used for maize, cotton, oilseed rape, and Roundup Ready soybean (Yelda Ozden, 2012).

3.1.2 Protein-based methods

Immunoassay methods are easy to execute, more rapid than DNA-based methods, have a low cost per sample, and do not require expensive equipment (Marmioli et al., 2008).

Enzyme-linked immunosorbent assays (ELISAs) are used to highlight antigen–antibody complexes when one of these molecules is labeled with an indicator reagent. The secondary antibody is most commonly labeled with radioisotopes, fluorochromes, chemiluminescent substances, and enzymes. There are three main types of ELISA: direct, indirect, and

sandwich. The antigen–antibody chemical reaction occurs on a solid phase (96-well plate) (McKenzie et al., 2000; Tan et al., 2013). Binding of the antibody and antigen produces a stable complex, which can be visualized by adding a secondary antibody coupled to an enzyme. Addition of a substrate for that enzyme will produce a colored complex, which can be spectrophotometrically evaluated. ELISA kits give quantitative results in several hours with less than 0.1% detection limits. ELISA kits are available for the detection of CP4-EPSPS protein from *A. tumefaciens* expressed in Roundup Ready soybeans (Fraiture et al., 2015; Santiago-Felipe et al., 2014).

Lateral flow immunoassays (LFIs, also known as strip tests or dipstick tests) use antibodies that are captured and immobilized on a support membrane. The membrane is dipped into the test sample and the sample will migrate up the membrane through capillaries. If the sample contains the protein of interest it will bind to the captured antibody on the strip, accumulate, and appear as a high-intensity band. These tests generally produce qualitative or semiquantitative results; however, the manufacture and accessibility of specific antibodies is time consuming and this method is not suitable for use on heat-treated samples. LFIs for the detection of a broad range of proteins used in the production of GM sugar, soybeans, cotton, canola, and beets are commercially available (Ahmed, 2002; Chen et al., 2016).

Luminex is a multiplex assay that allows the simultaneous measurement of multiple proteins in one well using a reduced sample volume. This technology produces results that are comparable with ELISAs but it has a greater speed, efficiency, and dynamic range. Furthermore, multiplexing technology is less expensive than ELISAs. This technology uses nonmagnetic (polystyrene) or magnetic kits with antibody-coated beads. Both types of bead are labeled with precise amounts of fluorochromes. The proportion of different fluorochromes results in a unique spectral signature of microspheres, which can be identified by the Luminex detection system (Marmioli et al., 2008; Tan et al., 2013). The binding of a distinct bead to a distinct monoclonal antibody permits the analysis of multiple targets in a single well. The polystyrene beads are generally more expensive and difficult to use in plates, as automation of the assay is more difficult. Magnetic beads are more sensitive and flexible than the polystyrene beads and they can be used with flat-bottom plates. This multiplex method can detect and quantitate proteins, RNA, and DNA if the beads are independently conjugated to specific nucleic acid samples. This method can detect more than 500 distinct targets in one sample and it is used to detect elements of soybean (p35S and EPSPS), rice (Cry1Ac), maize, and cotton (Lin et al., 2015; Yeaman et al., 2016).

Immunoblotting (western blotting) is a semiquantitative method that uses gel electrophoresis to detect proteins in a sample based on their size and electric charge. Following electrophoresis, proteins are transferred to a membrane to permit protein detection. The detection of target proteins involves two types of antibodies: first, a primary antibody specific to the target protein is added, then a secondary antibody, which is specific to the primary

antibody is used. The secondary antibody is labeled with a reporter molecule that reacts with a substrate to release a signal, which can be colorimetric, chemiluminescent, or fluorescent. This method is very sensitive when monoclonal antibodies are used and it can even detect different isoforms of the target protein. The disadvantages of this method are that it is time consuming, expensive, and requires expensive equipment. Western blotting is less commonly used in the routine analysis of GMOs, and is usually employed to confirm preliminary results generated by other detection techniques (Tan et al., 2013; Tzu-Ming, 2002).

3.2 Direct Method: DNA-Based Methods

3.2.1 Qualitative PCR

PCR is the most common strategy for detecting GMOs. This molecular biology technique amplifies a single copy (or a few copies) of a DNA segment to generate thousands or millions of copies of the DNA segment (amplicons). It is an easy and cheap amplification tool to produce sufficient DNA for downstream analysis techniques. PCR assays require a minimum number of target DNA sequences in the sample, and that the sequence of the target DNA is known. A critical step in DNA amplification is the extraction and purification of DNA from the sample. PCR-based tests used for the detection of GMOs sequences have been classified according to their level of specificity: (1) widely used sequences, such as P-35S (CaMV 35S promoter), T-35S (CaMV 35S terminator), T-Nos (terminator of the nopaline synthase gene), bla (β -lactamase), and nptII (neomycin phosphotransferase II); (2) sequences for detection of a specific transgene; (3) sequences for detection of construct-specific targets, and (4) sequences for event-specific targets (i.e., transgene integration sites) (Fig. 14.1) (Rosa et al., 2016; Wei et al., 2016).

An increasing number of GMO detection tests are based on a suitable primer pairs; for example, primers have been developed to detect GMOs in soybean (Roundup Ready RR), maize (MON810, Bt11, Starlink, NK603, and MON863), cotton (MON1445

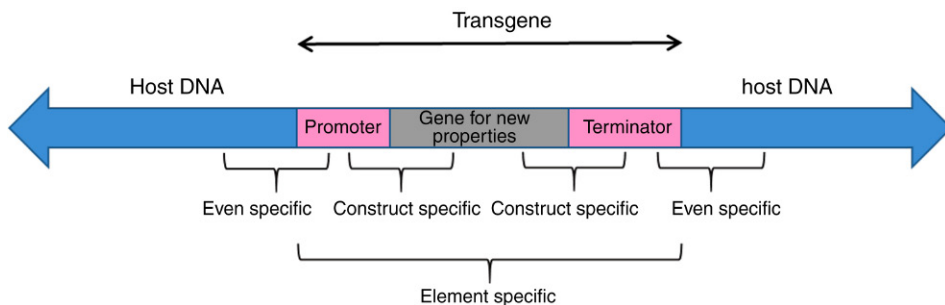


Figure 14.1: Diagram of a Typical Transgene Specific Construct. The Primer Pairs Targeting Particular Sequences Within the Transgene Integration Site are Illustrated as Event-Specific, Construct-Specific, and Element-Specific Sequences.

and MON531), oilseed rape, papaya, potato, rice, sugar beet, and tomato. Following amplification, the size of the amplicons are checked and visualized using gel electrophoresis. Amplicons may also be further analyzed using PCR-restriction fragment length polymorphism (using restriction enzymes that cut at specific sites of the amplicon), sequencing, or hybridization assays. The disadvantages of this method include the lack of multiplexing, its time consuming nature, the requirement for further analysis using gel electrophoresis, and that the results are only qualitative (Morisset et al., 2008; Nadal et al., 2006; Tan et al., 2013).

3.2.2 Quantitative real time PCR

Quantitative real-time PCR (qPCR) is the most commonly used method for detecting, identifying, and quantifying GM material in food products. This technique uses either SYBR Green or TaqMan chemistry (Navarro et al., 2015). qPCR uses primer pairs and fluorescent target-specific probes to detect the generation of amplicons in real time by recording the fluorescence generated by the fluorochromes attached to the amplicons. This technology is appropriate for both processed and unprocessed food matrices and it can detect amplicons of ~100 bp. Quantification is performed by comparing the cycle threshold of the two amplified sequences (the ΔC_t method) or by titrating against a standard curve. This method can be used as a multiplex reaction by labelling two or more amplicons with different fluorochromes. Major challenges for multiplex qRT-PCR are achieving sensitivity and reproducibility, and minimizing the risk of false negatives. The overlapping emission spectra of the fluorochromes used also produces technical difficulties. A novel triplex assay has been developed for the detection of Maximizer 176, Bt11, and MON810 in maize with a limit of detection (LOD) of 1% (Chaouachi et al., 2014).

The presence of a GMO is primarily assessed using a screening approach that targets the most common transgenic GMO sequences (i.e., p35S, tNOS, gat-tpinII, Cry3Bb, t35S pCAMBIA, and taxon-specific markers) using SYBR Green chemistry (Table 14.2). In this process a list of GMOs that are potentially present in the samples is established. These screening markers are included in the compendium of reference methods for GMO analysis and validated at the EU level following ring trials. Event-specific markers (i.e., those that target the join between two elements in the transgenic construct) or construct-specific markers (i.e., those that target the junction between the transgenic construct and the plant genome) are used at this stage to properly discriminate each GM event (Fig. 14.1) (Fraiture et al., 2015; Park et al., 2015). The SYBR Green chemistry for amplification detection has lower costs but is more susceptible to false-positive results and nonspecific binding than TaqMan chemistry. In TaqMan technology the hybridization between the probe and the target is very specific and it is these specific probes that are labeled with different fluorochromes. The specificity and high sensitivity of TaqMan technology means that subsequent data processing is not required; however, the synthesis of labeled probes for this technology is expensive.

Table 14.2: Representative examples of genes for targeting GMO (Ahmed, 2002; Anon, 2014; Barbau-Piednoir et al., 2014; Basak et al., 2014; Brod et al., 2014; Buermans and den Dunnen, 2014; Chaouachi et al., 2014; Chen et al., 2016; Di et al., 2014; Faria de Melo et al., 2013; Fraiture et al., 2015; Hamels et al., 2009; Holck et al., 2010; Hoorfar et al., 2011; Kiddle et al., 2012; Kruse, 2004; Leimanis et al., 2006; Li et al., 2008, 2015; Liang et al., 2014; Lin et al., 2015; Marmioli et al., 2008; Mazzara et al., 2013; Mbella et al., 2011; McKenzie et al., 2000; Morisset et al., 2008; Nadal et al., 2006; Nakaya et al., 2007; Navarro et al., 2015; Park et al., 2015; Randhawa et al., 2013; Rosa et al., 2016; Santiago-Felipe et al., 2014; Song et al., 2014; Tan et al., 2013; Tzu-Ming, 2002; Von Gotz, 2010; Wahler et al., 2013; Wei et al., 2016; Yeaman et al., 2016; Yelda Ozden, 2012; Žel et al., 2012).

Organism	Gene Symbol	Gene Name
Colza	ACC	Acetyl-CoA-carboxylase
Maize	ADH	Alcohol dehydrogenase I
Soybean	AHAS	AHAS fragment unique recombination from BPS-CV-127
<i>Streptomyces hygroscopicus</i>	<i>bar</i>	Phosphinothricin-N-acetyltransferases
Cauliflower mosaic virus	<i>CaMV</i>	ORFIII
Colza	<i>CRU</i>	Cruciferin
<i>Bacillus thuringiensis</i>	<i>Cry</i>	Gene encoding the δ -endotoxin
<i>Agrobacterium tumefaciens</i>	<i>CP4-EPSPS</i>	Enolpyruvylshikimate-3-phosphate synthase
Cauliflower mosaic virus	<i>CRT</i>	Reverse transcriptase from cauliflower mosaic virus
<i>Arabidopsis thaliana/A. tumefaciens</i>	<i>CTP2/CP4-EPSP</i>	Junction region between the chloroplast transit peptide 2 (<i>CTP2</i>) sequence from the <i>A. thaliana epsps</i> gene and the <i>CP4 epsps</i> gene from <i>A. tumefaciens</i>
Tomato	<i>FRUt</i>	β -Fructosidase
<i>Solanum tuberosum, Bacillus licheniformis</i>	<i>gat-tpinII</i>	Junction sequence between the glyphosate <i>N</i> -acetyltransferase of <i>B. licheniformis</i> and the terminator of the <i>S. tuberosum</i> proteinase inhibitor
Sugar beet	<i>GLU</i>	Glutamine synthetase
Maize	<i>HMG</i>	Major high mobility group protein
<i>Escherichia coli</i>	<i>hpt</i>	Hygromycin phosphotransferase
Rice	<i>LS28</i>	Choline kinase
<i>Corynebacterium glutamicum</i>	<i>LY</i>	Transition from <i>Zea mays</i> chloroplast transit peptide sequence for dihydrodipicolinate synthase to <i>C. glutamicum</i>
Soybean	<i>LEC</i>	Dihydrodipicolinate synthase (<i>cordapA</i>) encoding a lysine-insensitive dihydrodipicolinate synthase enzyme
<i>Brassica napus</i> , rapeseed, canola, and rape	<i>nptII</i>	Lectin
<i>Streptomyces viridochromogenes</i>	<i>pat</i>	Neomycin phosphotransferase II
Wheat	<i>PC</i>	Phosphoenolpyruvate carboxylase
Figwort mosaic virus	<i>pFMV</i>	Figwort mosaic virus promoter
Maize	<i>phy</i>	Phytase
Rice	<i>PLD</i>	Phospholipase D

(Continued)

Table 14.2: Representative examples of genes for targeting GMO. (cont.)

Organism	Gene Symbol	Gene Name
Soybean	<i>pNOS</i>	Nopaline synthase promoter
<i>A. thaliana</i>	<i>pRbcS4</i>	Ribulose 1,5-bisphosphate carboxylase small subunit promoter
Rice	<i>Pro</i>	Prolamin
Cauliflower mosaic virus	<i>p35S</i>	35S cauliflower mosaic virus promoter
Cotton	<i>RBCL</i>	Ribulose-1,5-bisphosphate carboxylase oxygenase
<i>Glycine max</i>	<i>SAD1</i>	Stearoylacyl carrier protein desaturase
	<i>SAMS</i>	Transition from S-adenosyl-L-methionine synthetase (SAMS) promoter to <i>G. max</i> acetolactate synthase (<i>gm-hra</i>)
<i>A. thaliana</i>	<i>tAHASL</i>	Acetohydroxy acid synthase large subunit terminator
<i>Pisum sativum</i>	<i>tE9</i>	Ribulose-1,5-bisphosphate carboxylase terminator E9
Common plant	<i>TLC</i>	tRNA-Leu chloroplastic
<i>A. tumefaciens</i>	<i>tNOS</i>	Nopaline synthase terminator
<i>A. tumefaciens</i>	<i>tORF23</i>	Open reading frame 23 terminator
Potato	<i>tpinII</i>	Inhibitor II terminator
Cauliflower mosaic virus	<i>t35S pCAMBIA</i>	Cauliflower mosaic virus terminator from pCAMBIA vector
<i>Oryza longistaminata</i>	<i>Xa21</i>	Xa21
Maize	<i>ZEIN</i>	Zein

qPCR is a rapid, flexible, and highly sensitive method that can detect low volumes of GMOs using multiplex methods (Mbella et al., 2011). However, the qPCR strategy is dependent on a number of additional factors. For example, this method is commonly restricted to one marker per reaction (or several if multiplex reactions are used), but additional markers need to be developed to permit full coverage of the increasing number of GMOs. This approach also only targets known sequences and therefore a negative signal can only assure the absence of a known GMO in the sample. A certified reference material (CRM) should be used to construct calibration curves for the proper quantification of GMOs; however, such CRM is restricted to only a few crop species (i.e., maize, soybean, rapeseed, cotton, potato, and sugar beet). The EU Commission recommendation 2004/787/EC stated that the measurement unit is defined as the percentage of GM target DNA sequences per target taxon-specific sequences calculated in terms of haploid genomes (plasmids certified on the basis of copy number could be used as alternative calibrants). New platforms have also been developed, such as the JRC-GMO-Matrix platform and the GMOMETHODS database, which integrate the positive and negative results determined using EU validated element-specific, taxon-specific, event-specific, and construct-specific assays for any tested food or feed matrix in order to accurately prognosticate the potential amplified GM events (Hoorfar et al., 2011).

3.2.3 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is an assay that uses stem-loop DNA constructs (DNA hairpins). This method uses four specific primers that recognize six distinct sequences on the sense and antisense strands of the target DNA. The isothermal amplification uses thermostable Bst DNA polymerase, which catalyzes strand-displacement synthesis. The final products obtained with LAMP are hairpin DNA molecules with several inverted repeats of the target structures with multiple loops formed by annealing between alternately inverted repeats in the same strand. The cycling reaction can accumulate up to 10^9 copies of the target in less than 1 h. LAMP is a highly sensitive method with a starting limit of six copies of the target sequence. This method is also highly specific due to the use of four distinct primers that target six different sequences. LAMP is a true isothermal method that does not require thermal cycling or heat denaturation of the template DNA. Therefore, it doesn't require expensive equipment and amplification can be performed using a heating block or a water bath. The amplification reaction can be directly visualized in the tube due to the use of fluorescent dyes and it is tolerant to several PCR inhibitors (i.e., polysaccharides). The disadvantages of this method are the complicated design of multiple primers and the inability to multiplex. LAMP has been used to detect viruses, bacteria, and fungi and it was found to be 1000 times more sensitive than PCR in the detection of *Streptococcus pneumoniae*. LAMP has been used to target the promoter of the cauliflower mosaic virus 35S, and different GMO sequences including, ADH, LEC, PLD, IVR, p35S pFMV, aadA, uidA, nptII, Cry1Ab, tNOS, pNOS, bar, pat, Cry1Ac, CP4-EPSPS, Cry2A, Cry3A, phy, Ms8, Rf3, MON89788, GTS 40-3-2, DAS-59122-7, MON863, TC1507, T25, *Bt*11, *Bt*176, MON810, B73-6-1, KMD1, and Kefeng-6 TT51-1 (Di et al., 2014; Kiddle et al., 2012; Randhawa et al., 2013).

3.2.4 Microarrays

Microarrays permit the detection of more than 250,000 targets in one assay and chips exist that can evaluate the expression of the whole genome. Microarrays have a higher throughput than PCR but a lower sensitivity. The principle behind microarrays is similar to that used in southern blot (DNA–DNA hybridization) and northern blot (RNA–DNA hybridization) techniques. It is based on the ability of complementary gene sequences to recognize each other and the ability to detect the presence (or absence) of DNA and RNA using fluorogenic or chemiluminescent detectors. mRNA extracted from a sample of interest is reverse transcribed to produce cDNA (or cRNA), which is fluorescently labeled and hybridized to an array surface precoated with target sequences. Expression levels are quantified based on the intensity of each fluorescent spot, which is directly proportional to the amount of mRNA present in the sample. Microarrays are useful for comparing expression levels between two different conditions (e.g., a plant with GMOs versus a plant without GMOs). Technological advancements in the late 1990s have led to the production of robotic systems that can position DNA fragments on glass media at a density impossible to achieve manually. Technological

advancements have continued at both the academic and commercial level. Current microarray platforms can differ in the types of probe they use, their design (i.e., nylon membrane, glass slide, or chip array), their technology manufacturing systems array (i.e., print or deposit), labeling, and hybridization protocols (i.e., one color or two colors). Microarray platforms use media containing cDNA or oligonucleotide arrays to evaluate gene expression. cDNA microarray systems are comprised of thousands of probes that generally correspond to PCR products in cDNA banks. cDNA microarrays require the selection of target sequences from public databases (e.g., Unigene, RefSeq, or GenBank), PCR amplification of genes of interest using specific primers, and purification of PCR products. The fragments obtained are stored in defined positions on the array surface by robotic deposition. Storage areas (spots) have diameters of 100–200 nm and these spots are separated by free zones of similar distances, reaching densities of 20,000 spots/cm². Microarrays have been used to detect GMOs in maize, cotton, rapeseed, and soybean (Hamels et al., 2009; Leimanis et al., 2006; Nakaya et al., 2007; Von Gotz, 2010).

3.2.5 Microfluidic dynamic arrays

qPCR performed in 96- or 384-well format with reaction volumes of 10–50 µL is still a low-throughput technique. Microfluidic dynamic arrays use a platform that allows a wider range of sample throughput. The advantages of this method are the significant savings in reagent consumption, pipetting, and hands-on work. The microfluidic chip consists of a nanoscale network of fluid lines, valves, and chambers that are controlled by pressure. Samples and assays are individually pipetted into inlets, loaded, and mixed in the chip. The chip is then ready for thermal cycling, which is carried out using regular PCR cycling. The chip is scanned and images can be visualized using dedicated software. This software can generate amplification curves for each reaction chamber within the chip. The high-throughput power of this system relies on the flexible chip setup, and chips can sustain 2304, 4608, and 9216 reactions in a single run. The high number of reactions and the low reaction volume (8 nL) means that it is necessary to perform a preamplification step to increase the sensitivity of the system and expand the number of analyzable target genes. These technologies can be used to detect protein crystallization, gene expression analysis, and single-cell gene expression analysis. Only a few papers have described microfluidic dynamic arrays for GMO analysis to date (Brod et al., 2014; Li et al., 2015).

3.2.6 Sanger sequencing

Sanger sequencing is a targeted sequencing strategy used to detect sequences of interest in large and complex genomes. This technique is very sensitive and can be used to sequence DNA fragments from a whole genome library (target enrichment sequencing) or a DNA library of PCR products (amplicon sequencing). Song et al. (2014) generated amplicons from maize using primers that targeted the endogenous maize gene, *Bt176* gene, *Bt11* gene, 35S/CTP4 construct, soybean endogenous gene, CP4-EPSPS element, p35S promoter,

and tNOS terminator. The authors performed this study in samples with low amounts of GM targets (1% of *Bt11* maize, 2% of GTS40-3-2 soybean, 2% of *Bt176* maize, 0.1% of GTS40-3-2 soybean, 1% of GTS40-3-2 soybean, or 0.01% of GTS40-3-2 soybean) (Basak et al., 2014; Holck et al., 2010; Song et al., 2014) and detected a minimum of 0.01% of GMOs in mixed samples.

3.2.7 Next generation sequencing (NGS)

NGS permits massive parallel high-throughput DNA sequencing. This method is rapid and offers the possibility to sequence many samples at the same time. These samples can be distinguished through the use of barcode-labeling. Targeted sequencing and whole genome sequencing (WGS) are the two main NGS strategies. WGS was used to analyze the LLRICE62 event and results were compared with the available reference genome of *Oryza sativa* ssp. *japonica*. Transgenic rice events, TT51-1 and T1c-19, and T-DNA sequences from the GM ax FP967 event were also investigated using this technology. The success of this method relies on the availability of good reference genomes. If a reference genome is not available, de novo assembly must be performed, which compares all generated results to identify overlaps. It is quite difficult to obtain good coverage of the plant genome because of polyploidy, repeated sequences, heterozygosity, and mixtures of different GMOs. However, NGS technology is a promising straightforward alternative for detecting and characterizing the sequences of GMOs in food matrices. Furthermore, the sequences obtained from unknown GMOs can be used to design new PCR markers. The relatively high cost and the requirement of bioinformatics expertise means that NGS has not been applied to all food or feed matrices at the current time (Buermans and den Dunnen, 2014; Liang et al., 2014; Wahler et al., 2013).

4 Validation and Standardization of Analytical Methods

Reference materials are required to obtain reliable results. This material is used for calibration, the assessment of sample measurements, and to assign values to samples. CRM should be stable, homogeneous, accompanied by a certificate, and established in interlaboratory studies. In the absence of CRM, standards validated by different laboratories can be used. It is necessary to establish LOD for each method, which is the lowest concentration or amount of analyte in a sample that can be reliably detected but not necessarily quantified. The presence of the analyte should be detected at least 95% of the time at the LOD, ensuring $\leq 5\%$ false-negative results (European Union Reference Laboratory for GM Food and Feed and European Commission 2008). It is also important to take into account the false-positive sample (the content of GMO that is below the LOD of the real-time PCR assay, at least 0.01% or 5 copies that tested positive in the qualitative analysis), false-negative sample (the content of GMO that is above the LOD of the real-time PCR assay, at least 0.01% or 5 copies that tested negative in the qualitative analysis), and the grey zone sample

(the content of GMO that is above the LOD of the real-time PCR and below the minimum sensitivity required for the qualitative method, $0.01 < x < 0.03$) (Lipp et al., 1999, 2000; Stave et al., 2000; Zagon et al., 1998).

5 Conclusions and Perspectives

qPCR remains the principal method for the standard analysis of GMOs in many laboratories. The GMO detection strategy or the availability of information about the targeted sequences may dictate that a specific technique needs to be used. To rapidly detect individual GM targets in a fully characterized GMO, PCR methods are absolutely appropriate. The simultaneously detection of several GM targets can be performed using microarray or Luminex. If only a part of the GMO sequence is known then strategies, such as NGS and targeting sequencing may be more applicable. The detection of GMOs through DNA detection will continue with the development of new technologies, such as microarrays, bio- and genesensors, and paired-end NGS. However, these techniques may reach the same level of progress as current techniques as the cost efficiency of replacing current GMO detection and quantification methodologies is an issue. Implementing common “biosafety protocols” is a major challenge for developing countries in all five continents as there is a lag phase in transforming and developing tools to deal with next generation biotechnology, in international methodology guidelines, and in standardized programs. Several research projects financed by the European Communion (i.e., DNA-TRACK, GMOCHIPS, QPCRGMFOOD, DNA-TRACK, GMOBILITY, and ENTRANSFOOD) have already explored a number of methods for getting the extraction, purification, and amplification of DNA from GM materials into a state of legislation for labeling substantially equivalent products containing GMOs, and reviewed and relieved the advantages of DNA analysis rather than gene product analysis (Marmioli et al., 2008). However, several issues remain to be solved, such as: (1) identifying and characterizing quantifiable endogenous genes considering the variation in the genome size and tissue ploidy level, (2) establishing DNA extraction methods from different matrices, (3) reference sampling, (4) theoretical and practical statistical analysis.

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Methods for Plant Genetic Modification

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1 Introduction

Genetic modifications (GM) are a group of methods that alter the genetic composition of a plant or animal to improve its nutritional content, shelf life, flavor, color, texture, agronomic properties, and processing characteristics. Plant genetic manipulation methods can be divided into those requiring in planta transformation or tissue culture (Darbani et al., 2008).

In planta transformation is a direct transformation method that does not require tissue culture and regeneration steps, thus avoiding somaclonal variation. This method can produce large amounts of transgenic plants that accumulate high concentrations of soluble proteins in a short time. However, the mean frequency of transformed progeny is relatively low and very variable (Darbani et al., 2008). Two *Agrobacterium*-mediated in planta methods (floral dip and vacuum infiltration) are usually used for both mono and dicot plants. Floral dip consists of dipping the floral elements in *Agrobacterium* culture, but it has low transformation efficiency and randomly integrates the foreign gene into the host genome. Vacuum infiltration produces transgenic plants through *Agrobacterium* infection of flowering plants. Transgenic plants are selected on media using a screening marker (Shamloul et al., 2014). The floral dip and vacuum infiltration methods are frequently used in agronomic crops (Solahil et al., 2016).

Only a limited number of species are transformed in the absence of a tissue culture-based regeneration system. The disadvantages of tissue culture-based transformation methods are that they are time consuming and produce somaclonal variation that can affect the qualitative and quantitative characteristics of the plant (Labra et al., 2004). The use of tissue culture-based methods can lead to unwanted genetic (e.g., point mutations and chromosomal aberrations) and epigenetic changes (e.g., methylation). Epigenetic changes are thought to underpin various well-known tissue culture phenomena (Smulders and de Klerk, 2011) and epigenetic silencing is considered for the silencing of transgenes. Epigenetic interference with the stability of transgene expression is reduced in plants whose plastid genome has been engineered for stable transgene expression (Moeller and Wang, 2008).

2 Methods for Genetic Modification

There are two main techniques for genetic manipulations: genetic and nongenetic engineering; however, there is not a dichotomy between genetic and nongenetic engineering as some mechanisms are common to both techniques.

Methods for genetic modification	Nongenetic engineering	• Simple selection (the selection of plants for continued propagation according to a desired phenotype)
		• Embryo rescue (the rescue of plants from hybrid embryos that cannot survive in vivo)
		• Crossing (obtaining a hybrid by mating sexually compatible individuals of different varieties)
		• Somatic hybridization (obtaining new hybrids through the in vitro fusion of protoplasts derived from different plants)
		• Somaclonal variation (genetic variability found among progeny of cells/tissue cultures in vitro)
		• Mutation breeding (the use of chemicals or physical mutagens to produce new plant varieties)
		• Cell selection (the isolation and cell-culture growth of cells selected for desired phenotypes that can be used to regenerate the whole plant)
	Genetic engineering	• Targeted manipulation of genetic material (e.g., cloning, viral vectors, microprojectile bombardment, electroporation, or microinjection)
		• Nontargeted manipulation of genetic material (random DNA mutations induced by chemicals or physical mutagens)

2.1 Nongenetic Engineering Methods

The oldest method of engineering consisted of selecting (over several generations) individual seed populations that met the desirable features (e.g., special phenotype or physical characteristics). This technique allowed the production of genetically homogenous seeds from heterogeneous ones and the production of new varieties. The lengthy selection process resulted in the generation of food plant species that were significantly different from the wild variant from which they were derived (Moeller and Wang, 2008). The method is still used but is now based on molecular analysis that identifies certain selection markers (mainly those that address resistance to plant diseases) thus allowing a more rapid selection of species with important agronomic traits.

Crossing allows researchers to obtain hybrids from two sexually compatible plants; however, this is a laborious method as recombination is random and requires a large number of hybrid intermediates to be produced before those with the desired features are obtained. Improved variants using this technique are currently in use. Interspecies crossing can be achieved either naturally (in particular conditions) or through human intervention and involves the integration

of genes from one species into the genome of a closely related species. Hybrids produced using this method have the characteristics of both parents. Cytogenetic manipulations were coupled with interspecific hybridization to transfer disease- and insect-resistance genes into crops. (Jauhar, 2006). There are several patents that have used interspecies crossing to obtain new varieties of plants (i.e., apomictic soybean varieties having a determinate growth habit, patent number 9210847/2015; tomato plants that exhibit resistance to *Botrytis cinerea* and have commercially desirable characteristics, patent number 8829280/2010; lettuce (*Lactuca sativa* L.) plants with resistance against the lettuce aphid *Nasonovia ribisnigri* (Mosley); and hybrid pepper plants resistant to geminiviruses, tobamoviruses, and damage by *Xanthomonas*). Chromosomal translocations can be performed to transfer large chromosomal segments between related species. Although the method has proved valuable for obtaining plant varieties, the length of the chromosomal segments also allows the transfer of some unwanted genes. Recent advances in chromosome engineering have facilitated the transfer of small DNA fragments and produced better results in the nutrition and food industries (Singh, 2011), mostly for genes that confer resistance to herbicides and insects (Yu et al., 2016).

There are cases where hybrid embryos produced from natural pollination between species (interspecies crossing) have not been capable of maturation. Embryo rescue technology (or embryo culture) is based on the insertion of the embryo in a special culture medium before seed abortion can occur and is used to breed parental lines with incompatible genomes (Mahgoub, 2015). The technology has been used to breed various crop species and has contributed to the production of useful species for the diet. The disadvantages of this method (i.e., the numerous selection processes, the inability to remove some undesirable traits, and the inability to use transgenics from unrelated species) required the implementation of modern technologies (Moeller and Wang, 2008).

Somatic hybridization (cell fusion) is a nonsexual genetic method based on the fusion of protoplasts derived from different plants. The removal of cellular walls through enzymatic techniques (i.e., degradation of polysaccharides by cellulase, hemicellulase, and pectinase) and the pooling of protoplasts (in the presence of a chemical/electrical fusogen) results in the production of a heterokaryon (Mahgoub, 2015) which has genetic material from both plant sources. This method overcomes the obstacles of hybridization pollination but not those related to chromosomal incompatibilities and is not currently used (National Research Council Committee, 2004) because it requires tissue cultures which may lead to genetic instability and somaclonal variation.

Somaclonal variation is an important source of phenotypic variation in plants and is caused by chromosomal rearrangements (that lead to spontaneous mutations) or epigenetic changes (induced by stress) during tissue culture (Mahgoub, 2015). Plant tissue culture refers to techniques that propagate plant cells/tissues/organs using a specific nutrient substrate. The occurrence of somaclonal variations had been attributed to numerous stress factors, including

explant preparation (e.g., wounding and sterilization), imbalances of media components (i.e., sugar and plant growth regulators, such as auxin and cytokines), and the in vitro culture environment (e.g., lighting and humidity conditions) (Krishna et al., 2016; Sato et al., 2011). Point mutations or chromosome rearrangements induced in somaclonal variation segregate, especially in the first generation. This technique can be used to improve plant traits (mostly for crops) and to select complicated traits (i.e., yield, soluble solids, sweetness, texture, or shelf life). No in vitro methods have been developed to date (Krishna et al., 2016). Selection failure can occur due to negative modifications, positive changes effected in a negative way, or instability of the changes after selfing or crossing (Karp, 1992). However, somaclonal variation is cheaper than other methods of genetic manipulation. Tissue culture is one of the most important steps in plant transformation as novel variants have been reported among somaclones, thus highlighting the effect of tissue culture on locations of the genome that are not accessible through conventional/mutation breeding. The studies conducted so far have made tissue culture systems available for a greater number of plants than could be handled by transformation. There is no need to identify the genetic foundation of the trait using this method, nor is there a need to isolate and transduce the responsible gene. Using cell culture to create somaclones avoids the risks of receiving chimeric expression.

Mutagenesis induced by chemicals and physical factors is used to induce random modifications in the genomes of plants. The randomness and nonreproducibility of these mutations makes the methods not useful for food. Ethyl methanesulfonate, calcium phosphate (Chowdhury et al., 2004), and lipofection (Kawata et al., 2003) are commonly used chemicals; electroporation, biolistics, vacuum infiltration, silicon carbide whiskers, ionizing radiation, and shock wave mediated transformation are commonly used physical factors (Rivera et al., 2014). Vacuum infiltration is used to facilitate the entry of some strains of *Agrobacterium* (under negative pressure) into the intercell spaces of certain cell types and has the advantage of low somaclonal variation levels as no tissue culturing is used (Rivera et al., 2014). The main application of this technique is the production of plant-derived vaccines for human clinical trials (Lai and Chen, 2012). However, induced mutagenesis can lead to deleterious mutations and has been used for more than 2000 varieties of crop in the United States.

2.2 Genetic Engineering

Genetic engineering involves the targeted change of a gene sequence to produce a specific result. In this regard, genes encoding proteins of interest are identified, isolated, inserted into a delivery system, and transferred into an appropriate host through complex processes known as recombinant DNA technology.

The methods of introducing DNA into plant cells can be divided into two major categories: indirect delivery through biological systems (via bacteria, usually *Agrobacterium tumefaciens*) and direct delivery using an intermediate host (usually biolistic and protoplast transformation).

2.2.1 Biological-based transformation systems

Biological-based transformation methods use plasmids, which are circular extrachromosomal double-stranded molecules of DNA found in bacteria, yeast, and some eukaryotic cells. These molecules are not essential for host survival but they can confer them some advantages in certain environmental conditions or over other organisms from the same ecological niche (Actis et al., 1999). Plasmids are frequently used as vectors to deliver genes into a host. The most common method for generating recombinant plasmid uses restriction enzymes and DNA ligases. In this regard, the donor DNA and the plasmid are cut by restriction enzymes at specific targeted sites (each type of restriction enzyme recognizing a specific DNA targeted sequence) and DNA molecules are combined by DNA ligases and a chimera DNA molecule containing both donor gene and plasmid DNA is formed (Griffiths et al., 2000; Lodish et al., 2003). The recombinant plasmid is subsequently introduced into bacterial cells where it replicates due to its autonomous replication capacity (Carattoli, 2013); plasmid vectors contain a replication origin that assures replication when initiated by host-cell enzymes. A selectable gene, usually for drug resistance, is also inserted in the plasmid vector structure to allow transformed bacterial cells to be selected (Griffiths et al., 2000; Lodish et al., 2003). Other method to generate plasmid vectors uses fusion polymerase chain reaction, in which primers that overlap different DNA fragments allow the combination of two different DNA molecules by their fusion during PCR. Vectors can also be obtained using different recombinatorial cloning systems that allow modification of the fungal genome (Schoberle et al., 2013).

Biological-based transformation systems mainly use *Agrobacterium*-mediated transformation in plants [e.g., *A. tumefaciens*-mediated (T-)DNA transfer]. *A. tumefaciens* is a natural genetic engineering instrument. It is a motile, aerobic bacterium that causes crown gall disease, and has the capacity to transfer its genome into the plant cell by tumor-inducing (Ti) or root-inducing (Ri) plasmids. Moreover, they contain genes encoding hormones and opines and assure the formation of a niche for *Agrobacterium* growth and proliferation. They also contain a region encoding proteins responsible for DNA transfer from the bacterium to the plant (Montagu and Zambryski, 2017). To use T-DNA as a vector, the Ti region is removed and the donor DNA is inserted between the border regions. This natural system is used in plant transformation, being responsible for the majority of GM plants on the market (Christie and Gordon, 2014; Gelvin, 2003; Ziemienowicz, 2014).

The mechanism of T-DNA integration is still not clear. T-DNA enters into the host as single-stranded DNA (Park et al., 2015; Rashid and Lateef, 2016). The integration system of T-DNA is activated by phenolic and sugar molecules that induce virulence proteins in the plasmid thus leading T-DNA into the host cell by VirD2 protein through the *Agrobacterium* type IV secretion system formed by VirB1–11 and VirD4 proteins. VirD2 is an endonuclease that cut T-DNA from plasmids and remains attached at the 5' end of the T-strand. The nuclear targeting of the VirD2/T-strand complex is mediated by importin α proteins which allows

the passage of the complex into the nucleoplasm through nuclear pores. Another protein implicated in the nuclear targeting of T-strands is VirE2, which also protects T-strands from nucleolytic degradation in the host cell (Gelvin, 2012; Rashid and Lateef, 2016; Tzfira and Citovsky, 2006; Ziemienowicz, 2014). T-strands are uncoated by proteolysis in the nucleus and converted into a double-stranded form by DNA repair machinery. The resultant intermediary form (ds T-DNA) is recognized as a broken DNA fragment and integrated into the host genome by an unknown pathway (Park et al., 2015; Rashid and Lateef, 2016; Tzfira and Citovsky, 2006).

Advantages of this system are based on the possibility to transfer one or more genes through direct cloning of cassettes into T-DNA and plant transformation with a separate helper (vir) plasmid; however, the system's have several disadvantages like specific targeting of cell nucleus and not organelles, limited transfer rate of DNA into monocots, and risk of transferring the insert to fungi. These disadvantages have led to alternative strategies based on P-DNA fragments (Darbani et al., 2008). Plant-derived (P-)DNA fragments replace *Agrobacterium* transfer T-DNA and have the potential to produce genetically engineered plants that contain only native DNA (Conner et al., 2007). Biological-based transformation systems also include viral-based transformations which uses plant viral vectors derived from geminivirus, tobacco mosaic virus, potato virus X, and cowpea mosaic virus (Gleba et al., 2014). Although these viral systems are highly expressed in plants (even at a systemic level) and have the potential for inducible expression, they are transiently expressed and show a lower infectivity for larger transgenes (Marillonnet et al., 2005). The most concerning aspect of viral vectors is their potential to induce posttranscriptional gene silencing, thus higher levels of transgene expression can be achieved via agroinoculation (Liu and Kearney, 2010).

The development of new technologies for the transient and stable expression of recombinant proteins in plants using viruses and bacteria outside of the *Agrobacterium* host range is extremely valuable in plant science. These techniques allow precise genome modifications (Hartung and Schiemann, 2014). Sequence-specific nucleases accomplish targeted DNA modifications by creating double-strand breaks in the genes to be transformed (Voytas, 2013). Double-strand breaks represent DNA lesions that can lead to cell cycle arrest and, if not repaired, to cell death (Ambrosio et al., 2016). Cells have developed different DNA repair pathways to ensure the genome integrity, the two most important pathways being homologous recombination and nonhomologous DNA end joining. These repair pathways can be further controlled to reach the desired sequence modifications (Voytas, 2013).

Zinc finger proteins (ZFP, ZFN) are a class of transcription factors, with Cys₂-His₂ zinc finger domain being one of the most common DNA-binding domains in the plant genome. ZFP are formed of a cleaved domain derived from FokI (an IIS restriction enzyme) and a binding domain. The binding domain of a ZFP wraps around the DNA molecule in the presence of a zinc atom, each finger binding with 3–4 base pairs (bp). ZFP forms a compact $\beta\beta\alpha$ structure

with the DNA molecule targeting a site of 9 bp (Maeder and Gersbach, 2016). To ensure double-strand DNA breaks, two binding sites of a ZFP located on the top and bottom strands of the DNA substrate in an inverted orientation, dimerize, and facilitate the nuclease domain activity. Since each ZFP from the dimerized complex requires two copies of 9 bp sequences in a tail-to-tail orientation, the specific recognition site must have 18 bp to induce cleavage of the DNA molecule (Durai et al., 2005).

The ZFP binding domain has a modular structure. To generate new functional endonucleases with different sequence specificity and to use them in genetic engineering, the binding sites responsible for sequence-specific interactions should be changed while maintaining the backbone of the ZFP (Durai et al., 2005). With regard to this, the fingers can be treated as independent modules and assembled in accordance with target sequences or constructed based on developed protocols and strategies (Carroll, 2011; Maeder et al., 2009).

Maeder et al. (2009) described a protocol of ZFN generation based on an “open source” combinatorial selection method called oligomerized pool engineering (OPEN). The ZFN engineering process can be divided into several stages in this process. First, the ZFN target sites within the gene of interest are selected using web-based software. The second step presumes the construction of B2H selection strains containing ZFN target half sites. Next, libraries encoding three-finger ZFN are created. The zinc finger arrays are constructed using OPEN, with each OPEN pool containing a collection of plasmids that encode three-finger proteins. Sequences encoding finger proteins are merged by overlapping PCR and cloned into an expression vector. The final step involves quantification binding activity of constructed ZFN in B2H reporter strains.

Transcription activator-like effector nucleases (TALEN) are formed of a DNA-binding domain specific to TALE proteins (original from *Xanthomonas*) and a DNA-cleavage domain (the catalytic fragment of FokI). The formation of dimers is essential for the induction of double-strand DNA breaks. The DNA-binding domain is formed of a tandem of 33–35 amino acids, followed by a single repeat of 20 amino acids. The amino acids in positions 12 and 13 of each repeat (repeat-variable diresidues) are responsible for recognizing and binding a single nucleotide base. There are currently several methods to engineer TALEN arrays. One limitation of TALEN is the presence of thymine in the 1 position directed by the constant N-terminal domain. The size and repetitive nature of TALENs challenges the stability of the delivery system (Cermak et al., 2011; Maeder and Gersbach, 2016).

The generation of a TALEN construct involves the selection of target sites within the gene of interest and assembling the repeat modules into intermediary arrays of 1–10 repeats in plasmids with a selection gene. The correct arrays identified using restriction enzymes and electrophoresis are then joined into a backbone to make a final construct (with ampicillin resistance gene for selection). The structure of the final construct confirmed by DNA

sequencing or digestion can be subsequently cloned into a vector of interest (Cermak et al., 2011).

Clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR associated systems (CRISPR/Cas) represent an adaptive immune system of bacteria that is responsible for protection against plasmids and viruses. Short sequences of foreign nucleic acids are incorporated into CRISPR loci, transcribed, and processed into crRNA, which forms a complex with transactivating crRNA (tracrRNA) and CRISPR-associated proteins (Cas) and induce the cleavage of targeted foreign DNA by Cas nucleases (Maeder and Gersbach, 2016).

There are three major types of CRISPR/Cas systems, type II being used for genetic engineering (Makarova et al., 2011). Type II CRISPR system components include a Cas9 protein, mature crRNA, and tracrRNA; for engineering purpose the last two components are fused into a single molecule of guide RNA (gRNA). The targeting specificity of CRISPR/Cas9 system is determined by gRNA, specifically the crRNA sequence. The single limitation of this system is the need for a protospacer-adjacent motif (PAM), which must be positioned immediately downstream of the target site. The gRNA/Cas9 complex associates with PAM motifs and forms a DNA–RNA heteroduplex at the target site, inducing the cleavage of target DNA by Cas9 (Bortesi and Fischer, 2015; Kanchiswamy, 2016; Maeder and Gersbach, 2016).

To generate a CRISPR/Cas system, a target-specific oligo is designed and annealed with a Cas9 specific scaffold oligo through the common complementary overlapping region. The resultant double-stranded DNA template is transcribed using the T7 promoter in the scaffold oligo structure. The functional gRNA is then purified from DNA (by treating the mixture with DNaseI) and from proteins, salts, and unincorporated nucleotides (using spin columns). The purified gRNA is introduced into a vector that also contains a Cas9 sequence and a selection gene and the structure of the final construct is confirmed by DNA sequencing (Ran et al., 2013).

Generated constructs can be delivered into host cells using physical or chemical means or bacterial systems.

2.2.2 Nonbiological-based delivery methods

Nonbiological-based methods of plant transformation include: (1) particle bombardment/biolistics; (2) electroporation; (3) microinjection; (4) chemical transduction; (5) silicon carbide fibers; and (6) liposome-mediated transfection (lipofection).

Microprojectile bombardment is also known as the biolistic method. Klein et al. (1992) were the first to report the delivery of DNA into plant cells using high-velocity microprojectiles. In this process, microprojectiles carrying donor DNA or RNA were accelerated and penetrated cell walls and membranes without killing them. This method allows gene delivery in many

subcellular organelles, fungi, bacteria, plant cells and animal cells and does not require a vector (Rivera et al., 2012).

Microparticles should be chemically inert, have a high DNA-binding affinity and be able to release DNA once inside the cell. They are usually made out of gold, tungsten or platinum and are 0.5–5 µm in diameter. DNA-coated microparticles are used as projectiles accelerate using a gene gun hitting a porous screen. Donor DNA is released after hitting the cells and it can integrate randomly into the host DNA (Brandenberg et al., 2011; Rivera et al., 2012). Different nanoparticle systems have been developed for donor DNA delivery, including mesoporous silica nanoparticles, calcium phosphate nanoparticles and polydendrimers. Calcium phosphate nanoparticles and polydendrimers can deliver donor DNA through coculture methods and do not need any assistance (Brandenberg et al., 2011; Rashid and Lateef, 2016).

A series of techniques were elaborated to deliver donor DNA into protoplasts. These methods are based on the totipotent capacity of plant cells (Vasil and Vasil, 1972), which makes the regeneration possible from a protoplast. Protoplasts (plant cells without cell walls) are obtained by enzymatic removal of cell walls (Zhai et al., 2009), and donor DNA (e.g., naked DNA plasmids) can be introduced using electroporation and microinjection or chemical transduction.

Electroporation induces the transient formation of micropores in the cellular membrane under an electrical field. Plant cells are transformed in protoplasts to ensure the access of donor DNA into the cell, which has been permeabilized by the application of an electrical impulse. Plant protoplasts can also take up other macromolecules (i.e., lipids and proteins) from the surrounding fluid. Electroporation is limited by the need of regeneration of the transformed cells and the limited transformation rate (Brandenberg et al., 2011; Rashid and Lateef, 2016; Rivera et al., 2012). Other techniques for protoplast transformation include microinjection, in which donor DNA is injected directly into the cell through a glass microcapillary-injection pipette. The transformation of oil palm protoplasts (fixed in an alginate substrate) by microinjection of an exogenous DNA showed only 14% efficiency (Masani et al., 2014), although other research groups have reported efficiencies of 20%–53% in tobacco (Kost et al., 1995; Schnorf et al., 1991). However, this method remains laborious and also requires special equipment (Brandenberg et al., 2011; Rivera et al., 2012).

Protoplasts can also be *chemically* transformed. The most commonly used technique is PEG treatment, which determines reversible cellular membrane permeabilization that allows donor DNA to enter into the host cell either as naked DNA or encapsulated into liposomes (Brandenberg et al., 2011). Poly-L-ornithine, poly-L-lysine, and dextran sulfate have also been used to increase the transfer efficiency while the use of spermidine protected DNA from shearing (Darbani et al., 2008). Other methods use plasmid DNA precipitation with calcium phosphate, the resulted complex invading through plasmatic membrane (Brandenberg

et al., 2011). Although these methods are easy, plant regeneration from transfected protoplasts has a reduced efficiency thus limiting its utility. Several other methods have also been described; for example, Chen et al. (2007) used short arginine-rich intracellular delivery peptides to deliver plasmid DNA into the nucleus of living plant cells without protoplast formulation.

Another method used for plant transformation is based on silicon carbide whiskers (silicon carbide fibers). Arshad group first describes this technique in 2008 and reported a transformation efficiency of 94% in cotton plants. The cotton transformants were stable and fertile and transgenes were transmitted in a Mendelian manner (Arshad et al., 2013; Asad et al., 2008). Silicon carbide fibers have high transformation efficiency in crops, such as maize and tobacco (Kaeppeler et al., 1990) as they can introduce small holes into cellular walls without affecting viability. This method is easy and rapid and consists of mixing (under vortexing) silicon carbide fibers, a suspension of tissue (e.g., cell clusters, immature embryos, or callus) and plasmid DNA. The efficiency of this method depends on several parameters of the target cell, the fibers, and the vortex characteristics. The simplicity of transferring silicon carbide whiskers with DNA into the cells makes this method very attractive; however, the time required for embryogenic tissue culture to generate fertile plants is similar to other methods using transgenes. (Petolino and Arnold, 2009).

Liposomes are spherical lipid bilayer vesicles of artificial origin, which can transfer the transgene into a target organism. Their transfer ability relies on the lipid bilayer fusing with the cell wall. Liposomes have several advantages that makes them appropriate vectors for gene delivery, such as their ability to protect transgenes against nucleases, their increased transfer efficiency, their ability to deliver to a variety of cell types, and their ability to deliver small organelles. (Gad et al., 1990).

3 The Role of Epigenetics in Plant Transformation

One of the issues raised by transgenic technologies is the epigenetic mechanism involved in the regulation of gene expression. Epigenetic mechanisms (e.g., DNA methylation, histone modification, and noncoding RNAs) modulate gene expression and play an important role in defending against pathogens. Studies focused on the unstable expression of transgenes showed that methylation mechanisms involving promoters correlate with chromatin alterations. Two types of transgene-silencing phenomena have been reported, which are determined by the position of the gene insertion in the host chromosome or the number of copies of the same or homologous sequence (Rajeevkumar et al., 2015). In homology-dependent gene silencing interactions between homologous DNA sequences leads to transgene silencing. When interacting genes display homology in promoter regions, silencing occurs at transcriptional level and is determined through methylation. The first breakthrough in this regard came from Matzke et al. (1989) who noted that transgene silencing in

Nicotiana tabacum was associated with DNA methylation in the transgene promoter (Matzke et al., 1989). Briefly, silencing was determined through the interaction between two promoter counterparts present in two T-DNA constructs, of which only one displayed methylation. DNA methylation of transgenes is also accompanied by other epigenetic changes, such as chromatin remodeling, which protects the transgene against host endonucleases. Moreover, subsequent studies in transgenic tobacco plants (T-DNA containing a cDNA of the potato spindle tuber viroid) demonstrated the contribution of RNA to de novo DNA methylation at homologous regions. Small RNA molecules are also involved in targeting homologous DNA sequences for methylation and for degrading through dsRNA formation. Small RNAs can not only contribute to silencing through target specificity (Meyer, 2013), but can also transmit the signal to other plant organs, which could lead to the silencing of undesired genes. Noncoding RNA-mediated gene silencing has been explored for the development of noncoding RNA-based GM plants (Ramesh, 2013).

4 Potential Risks Related to the Use of Genetically Modified Foods

GM may induce unintended changes that are associated with unwanted health effects. For this reason, the entire modified feature induced by the genetic modification (intended or unintended) must be tested for adverse health effects caused by the intake of a new substances or an intolerable dose of the substance. Detailed food composition assessments are determined using current analytical methods and DNA/RNA/protein profiling. Differences in composition must be correlated with their biological significance. To prevent adverse health effects, the unintended health effects of genetically engineered foods must be compared with the unintended effects of conventional genetically modified foods. Several human health hazards are associated with GM plants, such as the accidental introduction of a new allergen or toxin or antibiotic resistance.

Gene transfer from one cell type to another leads to the synthesis of proteins that were absent in the parental cells. The amino acid sequence of the novel protein structure is the main risk for developing food allergies to transgenic foods. The term allergy defines the pathological immune reaction to an antigen in a specific food component. Proteins are considered to be the main food allergens and their consumption may induce sequential skin reactions and alterations in the functioning of the respiratory and circulatory system that can lead to anaphylactic shock (Bernstein et al., 2003; Kramkowska et al., 2013; Ladics et al., 2011). Proteins obtained as a consequence of GM have allergenic potential if their sequence is homologous to an allergen sequence. At a global level approximately 2% of adults and 6% of children have food allergies. (Bernstein et al., 2003; D'Agnolo, 2005; Kramkowska et al., 2013). Special attention is given to allergies resulting from the consumption of transgenic foods. One example is the highly publicized Aventis case, which highlighted the adverse effects of genetically modified maize. The modified plant (StarLink) contained a

transgene that produces resistance to pesticides. The genetic information transferred from *Bacillus thuringiensis* to the maize cell induced the expression of the highly allergenic protein, Cry9c. StarLink corn was approved to enter the trade market exclusively as animal feed; however, shortly after commercialization the transgenic corn was found in human food. The dissemination of information through the media was followed by numerous reports of consumers experiencing symptoms associated with food allergies (i.e., headache, diarrhea, nausea, and vomiting) (Batista and Oliveira, 2009; Bernstein et al., 2003; Domingo, 2007; Kramkowska et al., 2013). Another example of a GM food allergy involves the production of soy enriched in methionine, an amino acid obtained by synthesis of a gene that was isolated from Brazil nuts. Walnuts and walnut plants are known to cause allergic reactions; therefore, the consumption of genetically modified soy represents a danger in people sensitive to nuts (Key et al., 2008; Kramkowska et al., 2013). GM foods that cause allergies are most commonly linked with the induction of novel genes and genes derived from organisms that have allergenic potential. In the case of Lucerne, the introduction of genetic material from sunflower seeds triggered allergies similar to those induced by the consumption of Brazil nuts (Kramkowska et al., 2013).

Another negative effect of genetically modified plants is represented by the cellular synthesis of toxic products, which can increase the activation of neoplastic processes. This can be exemplified when rapeseed oil derived from genetically modified plants was allowed to enter the market in Spain in 1983. The consumption of rapeseed oil led to the death of many consumers, although previous studies in rats had demonstrated the existence of adverse effects. Research into this phenomenon has involved several centers and the intoxication is thought to have been induced by toxic oil syndrome (oil contamination with aniline or aniline derivatives). Experiments by Suarez et al. (1985) showed no significant differences between rats fed for 2 months with oil contaminated with aniline and control rats fed with uncontaminated oil. Quero et al. (2011) also pointed out that genetic diversity is one of the factors involved in the toxic oil syndrome response (Kramkowska et al., 2013; Suarez et al., 1985). Negative effects of genetically modified foods were also reported in the United States in 1989. In this event transgenic tryptophan caused pain (mainly in muscles and joints) and even several deaths. Eosinophilia-myalgia syndrome was a reaction at L-tryptophan consumption. L-tryptophan is a dietary supplement and is usually used to treat insomnia or depression. In this case the toxicity was primarily related to the technological processes and purification of the L-tryptophan containing compounds (Kramkowska et al., 2013).

The health effects of transgenic foods are thoroughly investigated in animals. Different researchers have presented data on the harmful influences of MON810 maize (with pest resistance) on pancreas, intestine, liver, and kidneys in rodents (Kramkowska et al., 2013). Similar studies have shown that different varieties of GM maize (MON810 and MON863) that are resistant to insects, NK603, and the herbicide Roundup, can be toxic to the liver and

kidneys. This was confirmed in experiments where rats were fed for 90 days with 11 and 33% transgenic corn components; however, the study results were contested due to a small number of rats in the test group (80) compared to rats in the control group (4 times higher). In addition, toxic effects should have been evaluated over a longer period (i.e., 2 years) (de Vendomois et al., 2009, 2010). One study that was extended over a period of 2 years demonstrated that animals fed with maize NK603 and especially animals exposed to Roundup presented palpable tumors (Seralini et al., 2014).

Female rats also showed a higher sensitivity to foods containing toxic compounds. One possible explanation could be the low concentration of flavonoid antioxidants, such as ferulic acid (reduced by 16%–30%) and caffeic acid (reduced by 21%–53%), caused by overexpression of 5-enolpyruvylshikimate-3-phosphate synthase from *A. tumefaciens*, which provide plant resistance to Roundup (Seralini et al., 2012). Modulating effects of isoflavonoids on the estrogen receptor have been reported in previous studies (Duke et al., 2003; Seralini et al., 2012). Agglutinin and lectin synthesized in GM potatoes are toxic to mammalian growth and development. A diet rich in genetically engineered potatoes, which contains lectin gene expression from *Galanthus nivalis* (Domingo, 2007; Key et al., 2008) causes deregulated cell division within gastric mucosal cells; however, it should be noted that the effects are linked not only to the presence of the transgene, but also to other genetic factors. It should also be added that the described experiments involved a small number of animals and a short study duration (10 days) (Domingo, 2007).

Another aspect that must be mentioned is the risk of developing antibiotic resistance due to the transference of antibiotics resistance genes from GM organisms. Early biotechnological studies commonly used bacteria as markers to enable the identification of transformed cells. Regular use of therapeutic agents as transformation agents can lead to the transfer of resistance genes from physiological and pathogenic bacteria to digestive tract microbiota. Consequently, disease-causing pathogens can develop resistance to specific antibiotics. To avoid adverse health effects, it is recommended to eliminate antibiotic as selection markers (D'Agnolo, 2005; Kramkowska et al., 2013).

5 Conclusions

There are many methods for obtaining GM plants and each has its advantages and disadvantages in terms of productivity in plants, but most importantly, in terms of their effects on human health. Increasing the concentration of substances with beneficial health effects can be accompanied by adverse health effects (e.g., due to the production of antinutritive compounds, toxins, or allergens). Therefore it is necessary that plants with new characteristics be tested for extended periods of time using successive generations of animals to eliminate possible risks to human health.

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HANDBOOK OF FOOD BIOENGINEERING

VOLUME 6

GENETICALLY ENGINEERED FOODS

Edited by

Alina Maria Holban and **Alexandru Mihai Grumezescu**

Genetically Engineered Foods, a volume in the *Handbook of Food Bioengineering* series, aims to bring together the most recent progress in the field of Genetically Engineered Foods, emphasizing on current approaches, actual concerns in the biotechnological and food industry fields, and the most investigated and successful technologies. Also included is an overview presenting the current and future situation of Genetically Engineered Foods and their potential impact in resolving the emerging world hunger situation.

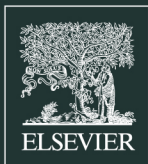
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